

nature

March 4, 1976

Getting down to business?

From the laboratory to the production line; a particularly difficult route, it seems, for British industry. First we look at the question of academics becoming industrialists.

OVER the past eight years the Wolfson Foundation has been supporting university-based research and development projects aimed at helping the national economy. Four million pounds has been made available for the period 1968–78, and sixty projects have been supported. A new round of grant applications is being solicited at present and a further £2 million will be made available to university groups with proposals which “have a real bearing on industrial production”. Some of the more successful of those who have earlier held grants from the Wolfson Foundation were in London recently to talk about their experiences.

Success can be difficult to measure. Some units have actually managed to generate sufficient income from royalties, consultancies and contract-work to guarantee them a life of their own when the Wolfson support runs out. Others have found that the work they were doing was (and this phrase came up more than once) too far ahead of its time in the industrial context; though this may not be unsuccessful in a broader context. But one field in which the admirable efforts of the Wolfson Foundation have as yet to bear much fruit is in persuading members of the academic community to stand full-square behind their own inventiveness and launch a company to take advantage of the business opportunities with which they are presented.

The foundation would no doubt argue that it has never been its role to encourage university staff to forsake the campus, nor was the money it supplied to be seen as venture capital. “We are not a merchant bank”, said Lord Zuckerman, one of the foundation’s trustees. And yet it needed only a little goading before he would add that academics had too little courage and were far too sessile when it came to considering industrial prospects. What he had hoped for was an increase in the number of small companies working from a technological basis of high quality.

Spin-off industry is, of course, a thoroughly American concept, and it would be foolish to ape the practice

simply because it comes from a country with a long record of academic–industrial rapport; after all, Route 128 has had its hard times. There are, however, some very positive reasons why universities should spawn more independent business.

Perhaps the most mundane reason is that if university and private business compete for the same work, it is possible for universities to fudge the overheads issue. This is not necessarily done malevolently—many at the Wolfson meeting were seriously questioning how they could make a realistic estimate of overheads—but the mingling of functions in a university environment is not, one suspects, a very healthy way of maintaining good university–industry relations. Nor, for that matter, is the use of students to provide the labour, in the long term.

A more important reason to encourage more spinning-off is the pressing need to demonstrate to students the role of private industry and the profit motive in a mixed economy. If he sees his teachers and advisers going through the motions of recommending to him the virtues of the private sector whilst themselves clinging to the security of the common-room, even though sitting on a fine business prospect, the advice can hardly bear much conviction.

There is much scope for misunderstanding in industrial–university collaboration. Each can so easily believe the other to be unreliable in delivering the goods. In particular the academic often sees “The Board” as a remote and fickle body, prone to abandon high technology at the slightest sign of recession or in the event of a take-over. Surely, then, the answer is often in the academic’s own hands: form his company, control his own board, then at least it will not be possible to blame anyone else if things go wrong—nor will anyone else reap the profits if things go right.

To the vice-chancellor the thought of losing good staff is obviously a worrying one, but there is no real reason why the long-term benefits should not be substantial. Much is talked and little is yet done about bringing scientific and engineering departments into closer liaison with industry for teaching purposes, both at the undergraduate and postgraduate level. Whom better to start such schemes with than managers of companies put together within, and spun off from, universities?



The development gap

... but what is NRDC doing? Professor S. D. Smith, who is both Professor of Physics at Heriot-Watt University and Chairman of Edinburgh Instruments Ltd, writes on the problem of support for development.

The Science Research Council (SRC) dispenses about £100 millions for research purposes each year. The community using it almost certainly contains the greatest concentration of academic brain power, but only a minor proportion of innovative, entrepreneurial and even practical ability, in the UK. But is there a mechanism to turn the practical results of this national investment efficiently into industrial products that could contribute to the economy?

Most people would, I submit, say no. One body representing academics, the Standing Committee of Professors of Physics, recently submitted a document to a Parliamentary Committee on Science and Technology stating that "most university investigators would prefer to invent nothing than something which might fall into the hands of the National Research Development Corporation". The organisation which automatically acquires rights for the exploitation of any activity currently funded by the SRC.

The appropriate development funding to take a device from a research laboratory to production line can be several times the cost of the original research. Certainly obtaining original research results is easier than making the equipment viable on a routine basis; for example, the laboratory research yielding the vertical temperature sounder known as the Selective Chopper Radiometer for the satellite Nimbus 4 cost about £40,000 while the development programme to engineer it for space cost nearer to £200,000.

One major problem is that NRDC funds for development extend only to about £5 millions per annum—in-sufficient, on the above arguments, to develop even a small proportion of "device-successful" research originating from the SRC. Even more important, NRDC officials themselves differ on whether or not the NRDC is there to encourage innovation and help British industry. But if its specific objective is not to help British industry, what is its purpose at all? Not only does modest development not seem to meet with favour in the NRDC; under the harsh financial terms the NRDC attempts to extract from companies

and investors, it seeks far ranging rights and then attempts to get its money back on *each* specific project by way of royalties at the rate of up to 12% per year on the unit selling price.

There are two immediate difficulties with this. Firstly, only about one out of ten projects may be *financially* successful, although a larger proportion may be technically successful. Secondly, the burden of a 12% royalty, being very large in proportion to the possible profit margin (probably about the same) in any competition with (in particular) American companies, can drive the selling price above a realistic level. I also suspect that the principle of trying to recover money *rapidly* on each specific project is wrong.

A related problem is that the NRDC only gives 50% of development funds. For both small and large industry today, this poses an almost insuperable problem of where to find the other half. A further condition invariably includes giving *all* rights and patents to NRDC: this is quite inconsistent with partial funding! The harshness of such terms make NRDC a worse prospect than a merchant bank—the more so because of the wearisome negotiations that drag on for months into years with innumerable wrangles with officials, accountants and so on. Thus in many ways it does *not* fulfil the role of financing truly developmental projects. There is only one saving grace; the NRDC shoulders the burden of loss in event of a failure. As I see it, the only way for a company to make a commercial success of an affair with the NRDC is to extract as much money from them as possible and then declare the project a failure.

So, how does a successful development occur at all? I believe that the British scientific instrument industry is almost in a position of not being able to progress without that element of luck or good fortune characterising all science and industrial development. The state of the laser industry and of the optical components industry and subtechnologies shows this clearly. The fact that things do happen is, I believe, quite often due to spin-off from some large project which carries with it a necessity for engineering and

technical development. The development of some component or system then takes place, initially through what I would call "tame orders". Such an order a company can cope with; it can carry out the work for a given amount of money. This must be responsible for much more development than the NRDC route.

Such procedure brings into question the role of agencies beyond the SRC/NRDC area and in particular the Ministry of Defence and so on, where the method is commonly applied. There are two disadvantages: this source of support is not generally accessible, as of right, as a route for development to companies working outside the "defence" area or by the university research community; and, second, it is dictated very much by the whim of current "defence" thinking. I believe that there is case for optimising the rules of the games for research and development expenditure in the direction of helping the development of devices when they lead towards industrial products. Opponents would argue that many of these things will not be economical anyway; but we are spending the money in any event with equally little economic reason.

Why don't we, as a matter of urgency and seriousness, optimise development routes? Some moves would require political initiation, but the terms of reference are not so difficult to envisage. With large projects (like the SRC's Laser Laboratory), some integration into British industrial effort should be attempted as a matter of policy. It must be wrong for the good of the nation to proceed into new technical areas independently of encouraging improved technology in the country. Then, instead of the NRDC, perhaps we require a new branch of the SRC, that can commission development work or pre-production orders and coordinate this activity with the pure science expenditure. Awards could be made and judged against a serious review of technical performance with the sanction of no more funds against inefficiency. Where industry is involved a shared support of the order 80% from SRC to 20% from industry might be right, combined with a liberal and flexible attitude to rights and patents.

To cries of "featherbedding", the answer is not to have no bedding at all. An energy gap may be a source of devices in semiconductors; a development gap is no source at all. This nettle must be grasped at the level of politics.

Speaking up for the telephone

Next week sees the centenary of the telephone. Below, a look at telecommunications in Britain

IT MAY be going too far to suggest that, in telecommunications, the pace of research and development may have all but outstripped our capacity to appraise and utilise it. But now more than ever (and in telecommunications more than anything) it takes specialists, in electronics and electrical engineering, to comprehend the intricacies, if not the implications, of the astonishingly broad and complex range of telecommunications "breakthroughs" in the 100 years since Alexander Graham Bell spoke seven words to his assistant on the first telephone.

Telecommunications is widely recognised in Britain as lying firmly within the public domain, being the nuts and bolts of a public service provided by the Post Office, a nationalised undertaking. The Post Office itself acknowledges this; so too does the government. And most people in Britain expect it. Yet the reality looks rather different, for the boundaries of the telecommunications industry are far from coincident with those of the British economy's public sector.

The Post Office, formerly the GPO but made a corporation in 1969, has long had a monopoly of the country's telecommunications services. The assets of the system it runs, which is the third largest in the world, are valued at over £4000 million; the Post Office is the country's biggest employer, with almost 250,000 of its 430,000 employees in telecommunications; investment in the year 1974-75 totalled £788 millions, of which 76% (about £600 millions) went on growth and the rest to maintaining and improving the existing system. The figures are enormous; but they form only part of the UK telecommunications picture.

Quite as important is the Post Office's position regarding procurement. In supplies it has nothing like a monopoly. Indeed, just last year, the Post Office produced a document containing recommendations on whether it should manufacture "in-house" more of the substantial amounts of equipment, worth hundreds of millions of pounds, which its existing suppliers produced for it. The question highlighted the network of links between the Post Office, a public corporation, and its main suppliers, all private enterprises with famous names—Plessey, GEC, Pye TMC and Standard Telephones and Cables (STC). A few months later, the nature of those links was further highlighted by announcements from all of these companies of

impending mass redundancies as a consequence of a cut-back by the Post Office in its projected orders for telephone exchanges, transmission equipment and telephone sets.

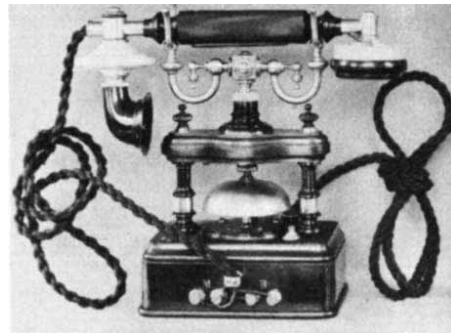
The reasons behind this upset were also revealing. They related, at least in part, to a 30% reduction in the Post Office's estimate on orders for 1975-76 and 1976-77 compared to its estimate a year earlier; this reduction was in turn a reflection of declining demand in the face of rising costs which the Post Office found itself obliged to pass on under the UK government's rediscovered "profitability" regime for the public sector. But the need for redundancies would have arisen anyway, since it was also the result of the design and labour-saving manufacture of electronic exchanges being introduced to replace more conventional electromechanical exchanges and so provide the basis for a massive modernisation programme for the UK telecommunications system.

Reactions to these redundancies also showed the sort of economic factors at work, but revealed political ones too, for the additional unemployment at a time of deepening recession naturally evoked deep emotions. Calls for the introduction of the electronic exchanges to be postponed and for old order levels to be restored, if successful, would have given the Post Office too many of the wrong exchanges which it could not really afford anyway (unless it passed the costs on).

Research and development

That new technologies as well as the plight of the economy are responsible for these cut-backs is just one indication of the central role which research and development plays in British telecommunications. There are others, of course, but a glance at the Post Office's books is sufficient to confirm its importance. In 1974-75, expenditure by the Post Office on research and development climbed fully 30% in value to £28.9 millions. Even more striking, about 93% of this (£27.1 million) was devoted to telecommunications, divided as follows: 59% (£16.1 millions) on "new systems", 25% (£6.8 millions) on "existing systems and related advisory services", and the rest (£4.2 millions) on exploratory research.

A Science Research Council publication released in 1974, surveying current research activities in the UK, says Post Office research and development then involved "some 3,500 staff, of



whom about 1,000 are qualified scientists or engineers". The Research Department, now located at Martlesham, the new research centre opened last year, conducts exploratory research into new systems and services as well as the technology, materials and devices to back them up; the Development Department elaborates this work, which focuses chiefly on "switching" (exchanges) and transmission.

Important as it is, however, the Post Office's teams are not the only ones in operation even amongst the public corporations. Work is also done by both broadcasting networks (the BBC and the IBA) and by British Rail. Furthermore, there is the mostly-secret Ministry of Defence activity, through the Signals Research and Development Establishment, the Admiralty Surface Weapon Establishment and the Royal Aircraft Establishment; and the SRC has its own Appleton Laboratory. Each of the Post Office's main suppliers—major companies all—also has sizeable research facilities, on a scale similar to that of the Signals Research and Development Establishment. The work done in these is, the SRC says, undoubtedly influenced by the Post Office, because it is their major customer; between them, in fact, they share several hundred million pounds worth of Post Office business annually. Finally, there are the universities and polytechnics, whose work involved (in 1972-73) 526 people and an expenditure of almost £350,000, the greatest amount coming from the public sector.

The research net is thus spread fairly wide. But what is caught in it? Practically everything conceivable and more besides, if such breakdowns by establishment as are available are anything to go by. While the jaundiced eye might cynically view those research efforts which are made glamorous by publicity-conscious promoters as mere attempts at self-justification, it would probably still focus on the extensively-publicised telecommunications work of the Post Office; after all, the telephone service remains the most direct contact with the world of telecommunications for the large majority of people.

Take the humble telephone set itself. In concept the instrument has changed little: but in a new microphone, sound

generates an electric signal from a plastic diaphragm with a built-in charge which is boosted by a transistor amplifier. Do the problems of general application, which are large, make such time, money and effort worthwhile? Professor Merriman, Senior Director (Development) with Post Office Telecommunications, has explained that the benefit of such devices lies "in their implied potential for modifying the balance of investment" between telephone sets and their lines to the local exchange. Similar economic arguments are deployed regarding development of the lines themselves. But the chances of reducing costs, without a "fundamental" change in technology, are, he has argued, "reaching an asymptotic limit".

Transmission and exchanges

It is precisely such a change which offers the possibility of transforming the main transmission systems, for here research and development has progressed enormously, particularly in the last ten years. Two entirely new forms of transmission system are being developed which virtually outdate the now-customary coaxial cable and microwave systems.

One uses "optical fibres" and transmits signals in the form of light pulses down extremely thin strands of glass fibre. They can carry more messages further and more cheaply with fewer amplifiers than conventional cables. The other uses "waveguides", and is described by the Post Office as "a kind of piped microwave radio"; in it, signals are borne on waveforms down a copper pipe. In both cases digital transmission is involved, where signals are sent as a code of *pulses*, obtained and retranslated through what is known as pulse code modulation, rather than in the customary analogue form, where electrical *waves* corresponding to sound need greater boosting.

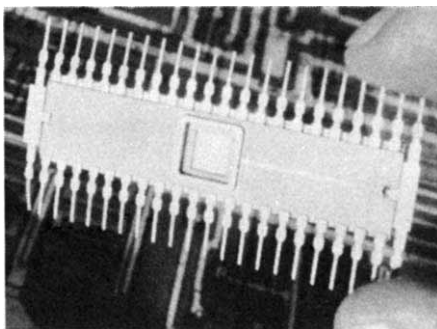
But perhaps most attention, certainly in the case of the Post Office suppliers, is being devoted to "switching", or exchanges. The key electronic development which is outdating electro-mechanical arrangements is the integrated circuit, or "chip", hundreds of which are no larger than a pin's head: made of semiconductor, these will replace the large electromagnetic relays: linked to form "processors", they could control central as well as local exchanges. The TXE2 is smaller and uses more basic technology than the TXE4, the electronic exchange developed for large local exchanges of up to 40,000 lines. For the suppliers they offer tremendous export potential, particularly with respect to countries establishing whole new telephone systems.

Switching now represents something like a third of the Post Office's overall

investment and is probably the most substantial element of the telecommunications industry's annual production. With the refinement of the electronic exchange, the development path for Britain's telephone system seems fairly clear for the rest of the decade. But it is the result of decisions taken over a period of some 15 years. Moreover, further developments in switching technology are anticipated, and soon. Their application demands foresight too; but it also demands consideration of the precise manner in which they will fit in to technological changes in signals and transmission. That means one thing if costs are to go on being reduced in the 1980s: as Merriman has put it, future switching technology "inescapably must be conceived in the context of total systems development and as part of the progressive move towards all-digital, microelectronic, software-controlled systems".

Which introduces what the Post Office calls its "System X", the catchword it proudly uses for overall modernisation of Britain's telephone system. The catchword is necessary: any explanation of it descends quickly into the jargon of systems analysis. The basic idea seems to be that a comprehensive strategy laid now by the Post Office and the industry for the future will, over the years as it is introduced on a larger and larger scale, plan and coordinate the direction of research at all levels, anticipate developments in advance which might in turn be incorporated into the strategy, and generally ensure balanced development and progress.

If the money is there. Thus far, as the figures themselves show, telecommunications has not had it all that bad: few other high technology sectors can point to coherent plans for the 1980s so confidently. And they do not relate only to the telephone system: the list of services now or soon to be provided includes radiopaging (the doctors' "bleepers"), Viewdata (up-to-the-minute information on the telly at the push of a button), Confravision and Viewphone (long-distance conferences between individuals who can see each other and exchange papers) and Fax, or facsimile transmission (a



Integrated circuit for electronic exchange

telex-photocopy combination). Even within the realm of telephony itself, improved international dialling and telex facilities, additional use of radio and satellites, automatic dialling devices, push button phones—all are held out, and the list goes on. Fourteen years after Telstar, in fact, space technology still represents a new frontier, and the Post Office has a share in a total of seven satellites.

In Britain, telephones have until recently proved an enormous growth area, and even last year's downturn was then regarded by the Post Office as purely temporary. There are now around 21 million telephones in the whole country. The Post Office argues that real costs to the consumer have fallen over the years, and have done so because of foresight over technological developments in transmission and exchanges. The experts, in telecommunications as in other comparable fields, wish to see the progress made thus far continue at the same if not an increased pace—partly, the cynics would say, to keep themselves in business, though also to supply a continuing demand at a reasonable cost. But projections for the hopefully rosier future can be wrong in an inflationary era. Moreover, the pace of well-financed development being what it is, if demand for various services does grow, even System X could be outdated by the time it is fully installed.

It is precisely this sort of consideration which highlights what many people regard as the real dilemma of telecommunications in Britain 100 years after the telephone: the inevitable duality of a rapid growth in telecommunications knowledge, on the one hand, and, on the other, the lengthy periods needed for appraisal and implementation. Decisions on the direction of research, on appreciation of results and on their application—the argument runs—consume inordinate amounts of time which the additional element of public accountability tends to lengthen still further. It is an argument used to press for greater competition in telecommunications services and to encourage less duplication of research. Others would like to see less research altogether and more grist to the consuming public's mill.

The end result is a mixture of efficiency and inefficiency, profit and loss, bitterness and sweetness: less something Britain can afford than something she cannot afford to be without—and a far cry from those days over a hundred years ago when those who could conceive the idea of transmitting a voice over wires nevertheless thought it would be of little practical value. □

AAAS

Making the annual pilgrimage

Seven years since it last graced Boston with its presence, the American Association for the Advancement of Science (AAAS) returned there a fortnight ago for its 142nd annual meeting. Roger Woodham reports on a heady week during which some Americans admired their national science effort—and others took it to task

MINORITIES in science, the Viking lander mission to Mars, the inevitable questions surrounding energy policy in general and nuclear power in particular, the views of organisations like Science for the People on everything from genetic manipulation to the busing of schoolchildren . . . this is a selection of topics which is at once typical and atypical of the Boston AAAS meeting. Atypical, because few if any of the 5,000-odd attending the meeting will have found it possible to draw all its diverse threads together; but typical, because it represents in a broad-brush fashion the tenor of the whole meeting. Some impressions:

- Allied to the whole question of opportunities for minority groups in science—consideration of which included papers like "Spanish-surname Americans in the sciences"—was the matter of women in science, or rather the lack of them. This was the subject of an unusual morning session on Great Women in Science, attended as might or might not be expected by 150 women and 10 men. The message was clear—either, like Margaret Burbidge of the University of California (who has recently been voted Woman of the Year in California), they had been actively encouraged from an early age, or, like Mildred Dresselhaus of MIT (one of three women members of the National Academy of Engineering), they had simply "stumbled into science and engineering".

When it comes to recognition of female scientists, it transpires that the National Academy of Sciences only does slightly better than its engineering counterpart. It has 21 women members at present, out of a total of 1,144, and has only ever had 26. But it can claim to have done better than the Royal Society of London in electing its first woman member in 1925 rather than 1948. Equal recognition, the meeting concluded, is as much (if not more) of a problem for women as equality of opportunity.

- One recurrent theme was concerned with the tying together of federal government decisions on tax and economic matters, research and develop-

ment, and regulatory policy. Dr William D. Carey (Executive Officer of the AAAS) contended that the burgeoning of regulations involving industry had led directly to general caution on the part of innovative industrial concerns and the handing down of cautionary decisions. Ninety per cent of industrial research and development is defensive, not imaginative, he declared.

Taking a broadly similar line, Dr Derek C. Bok, President of Harvard University, made remarks that will be echoed by his counterparts on the other side of the Atlantic. He complained that "the government has felt constrained to make painful choices about research priorities; choices that demonstrate the power of the purse to control the growth and shape of the universities". In times of money shortage, he went on, "the needs of institutions of special quality are readily dismissed as elitist and unnecessary". Crash programmes for this or that are all very well, but by focusing attention so heavily on immediate national problems, public officials run the risk of restricting 'advances in fundamental knowledge that will leave us ill-prepared for the crises yet to come'. Echoing Dr Carey, Dr Bok said that declining funds threatened the most novel risk-taking forms of science.

- No fewer than fifteen individuals directly involved in the Viking Mars programme—and one Congressman, Don Fuqua, known to be well disposed towards space science—contributed to a day-long seminar that not only mapped out what the mission hopes to achieve, but reflected, quite soberly, on what may happen next.

Viking, or rather the Vikings, for there were two identical spacecraft launched within a few weeks of each other in October last year, go into orbit around Mars on June 19 and August 7. And as Dr James S. Martin, the Viking project manager, put it, there's no flexibility in that date, whereas the patriotic notion of landing Viking 1 on Mars on July 4, at a place in the Martian Northern Hemisphere appropriately called Chryse ("hope"), could be scrapped if the information sent back from Mars orbit suggested that some other time or place for the landing would be appropriate. Viking 2 is scheduled to land on September 4 (in a different place) but again the Viking 1 lander and the part of it left in orbit would be helping to update Viking 2's final plans.

Everyone seemed to be agreed that the future for the space sciences would

in large measure be shaped by the results that come out of the Viking project—not only the photography of the landscape, by cameras capable of a resolution of 2 to 4 millimetres, but also the outcome of scooping up Martian soil and feeding it on a "rich organic soup", among other things, to see if there are any signs of life.

If there were, that might also be the signal for renewed activity in the search for extraterrestrial intelligence—another seminar topic which saw AAAS participants packed in the aisles. The idea of spending \$10,000 million on Project Cyclops, a giant 'listening' programme, is one that is pretty well buried, but more modest schemes to carry on and expand the listening work of the Arecibo radio telescope would undoubtedly blossom if Viking struck lucky.

Assuming some middle-of-the-road outcome for the Viking project—which certainly means no photographs of footprints—and bearing in mind the constraints of the NASA report *Our-look for Space*, which lays down those classes of project that should be attempted—what alternative ways ahead are there? More Vikings of the same kind, Vikings that can be mobile on the Martian surface, Vikings that drop penetrometers from orbit (instrument packages capable of burying themselves hundreds of metres down in the right conditions and sending back data such as temperatures), Vikings that can return a shovelful of Martian soil to Earth—all these are possibilities, as is further collaboration with the Soviet Union. But what everyone seems to be ruling out is landing a man on Mars, at least before the year 2000. That would undoubtedly violate NASA's guidelines for space research in the last twenty years of the twentieth century.

- Science for the People—a radical group that can see very little right in American science, it seems—has almost made it into the Establishment of the AAAS. During the past five years or so it has had a tolerate-hate relationship with the association at annual meetings, sometimes involving the police; this year it had its seminars formally included in the AAAS programme. Science for the People will snipe at anything—including Boston's school busing problem and the cost of electricity—but at the Boston meeting it was mainly addressing itself to topics like cancer research. The group asked, in one of its sessions, for example: "Why has so little effort been devoted to the problem of preventing cancer caused by occupational and environmental agents, when it is widely recognised that 80 to 90% [sic] of all cancer is caused this way?" □

THE GAMBIA

More the people than the place

Dr R. S. Bray, Director of the Medical Research Council laboratories in The Gambia, looks at life and work there.

THE 1975 balance sheet for medical research in The Gambia shows a reasonably healthy credit. Not everyone wants to do their medical research in a tiny republic stuck out in the savannah of West Africa, but there are compensations. The research is directly about people: organisms and medical phenomena are in people not guinea pigs, and epidemiology figures large. For this you need not merely people, you need nice friendly people, and an understanding population helps to make this the Kingdom of Heaven to an epidemiologist.

The Gambia's people have a willing belief in the delights and benefits of medical research and a considerable dignity to abash the over-assertive. When it came to importing a South African worker to do some haematological investigations, it was with some misgivings that the Ministry of Health was approached about his entry. But—"Bring him in," they said, "we'll show him how nice we are". And they did: he came back the following year and is now thinking of teaching them Rugby!

There are some drawbacks, of course. The rainy season can be tiresome, though there's lots of lovely organisms rife. Distinct seasons do make epidemiology interesting, but it's hard to work full-time on parasites which insist on disappearing by February and not reappearing until July. And of course we are remote. The cut and thrust of one's peers is sorely missed—each researcher tends to be working on a sufficiently different subject to be able to make confident statements based on shaky premises with never a demur heard.

Efforts continue to be made to get onto good terms with The Gambia's neighbours. Visits to Dakar are regular for such essentials as liquid nitrogen and frog's legs. But efforts to revive some institutional form of anglophone West African co-operation in medical research move slowly. The giant to the south—Nigeria—looms over all calculations. They have their own organisations, and the knowledge that they will foot the larger part of the bill for regional co-operative efforts doesn't impel them precipitously into such endeavours. But some concrete results have emerged. A collaborative investigation on malaria and the oral contraceptive pill was initiated between the

Komfo Anokye Hospital, Kumasi, Ghana and the MRC, Gambia with IPPF acting as midwife. Equipment has been another productive source of co-operation. It isn't worth £15,000 (for a cobalt 60 source) to irradiate some malaria infected mosquitoes occasionally, so they are taken to Accra where the Ghana Atomic Energy Commission's machine is used with their enthusiastic co-operation.

Malaria figures large as always in medical research. It is still uncontrolled in Africa though the easy availability of antimalarial drugs and domestic sprays is having an effect in and around cities. The sexual forms of the malaria parasite are being studied to see how and when it arises. The major gaps in the knowledge of African malaria are the reasons for the slow and gradual acquisition of immunity, but techniques for exploration of this phenomenon are hard to come by. Work is going on to investigate malaria in the first six months of life and in pregnant women, where variations in immune status may offer some clues. Vaccination is on the horizon, and work continues on the techniques necessary for the collection of suitable antigen. Work is also done on filariasis and the onset of elephantiasis, schistosomiasis and the longevity of the worm in the face of acquired immunity, the effect of various diseases on cellular immune processes, malnutrition and the contribution of disease to the onset of marasmus, the manner in which female mosquitoes find their hosts, the contribution of mycotoxins to primary hepatoma and on the susceptibility of N'Dama cattle to trypanosomiasis.

And of course there is the grandest tropical health study of them all—Ian McGregor's 26-year-old survey of 4 rural communities in West Kiang district. This unique work brings together 26 years of morbidity and mortality data, growth statistics, endemic and epidemic disease information, serological results and medical genetics. When eventually collected and collated it will form a standard of reference for medical demography and for medical genetics unsurpassed in Africa. Already the information spun off has been of enormous importance.

Always looming, however, is the fact that 4 to 5 of every 10 children born in Black Africa die before adolescence. When projects are designed, this fact can encourage, even justify—for if an experiment works which brings the disappearance of this disease forward one month, 100,000 lives might be saved. A sobering thought. □

IN BRIEF

JET lag

EEC efforts to take the Joint European Torus (JET) fusion reactor from the design to the construction and experimental stage were still stuck firmly in the political mud last week when community ministers failed to agree on a site for the project after a 16-hour tussle in Brussels. There was agreement on building and financing the project, but national opposition to an earlier Commission finding in favour of the site at the community's joint research centre at Ispra, northern Italy, forced an impasse which the ministers finally sought to resolve by establishing a new committee; this will investigate the relative technical advantages of the contending sites and decide whether experience in fusion research and plasma physics is relevant in choosing between them. Ministers are due to meet again in mid-June, over six months after the next five-year stage of the £96-million EEC fusion research programme should have begun.

Nuclear trade measures

In Washington, a Senate sub-committee on arms control heard last week that the "Group of Seven" nuclear exporting nations—Britain, Canada, France, Japan, the Soviet Union, the United States, and West Germany—have agreed still-secret measures to extend safeguards against the misuse of exported nuclear technology. Some clue to the nature of the pact comes from new US Government principles which require foreign recipients of nuclear facilities, equipment and materials to comply with the internationally accepted safeguards already laid down by the International Atomic Energy Agency (IAEA), and to demand identical assurances from any country subsequently handed similar technology. Recipients will also be restricted from using imported facilities for explosive purposes, whether peaceful or not, as a result of the new US policy.

Serendipity in science?

"There is nothing more practical than a good theory", said Mr Brezhnev, addressing the Twenty-Fifth Congress of the Communist Party of the Soviet Union last week. These words, reiterated by Academician Anatolii P. Aleksandrov, President of the Soviet Academy of Sciences, were apparently intended to justify expenditure on basic research in a Five Year Plan which urges that science should be made cost-effective and more closely linked to production. Citing examples ranging from nuclear physics and MHD-generators to cytology and genetics,

Aleksandrov stated that basic research "radically changes technology, leads to the appearance of new materials, and opens up possibilities of using new, often unexpected phenomena, which have no connection with the original field of research".

Dragon reprieve?

The death-throes of the 15-year old Dragon high temperature reactor project at Winfrith in the UK may last longer than expected if a proposal from the European Commission in Brussels is approved. The Commission wants to see the team kept together until the end of the year, mainly to allow the results of the whole project to be evaluated, but also, in turn, to

leave the way open for any possible but unlikely resuscitation. The proposed maintenance would cost about £1½ million.

BNFL complication

As British Nuclear Fuels Ltd awaits cabinet approval for its controversial Japanese nuclear processing deal, there are reports of French moves to break into the contract. Britain, being a member of the reprocessing group set up with France and Germany known as United Reprocessors Ltd, is obliged to discuss the French bid for a possible 50% share in the contract, or risk competing with France for the entire deal, worth up to £600 millions over the decade following 1980.

Dumping decision

Whatever the outcome of the BNFL negotiations, Britain is to continue dumping low activity material in the deep ocean. In a House of Commons written reply last week, the UK Energy Secretary Anthony Wedgwood Benn stated that desirable disposal rates of the "low activity plutonium contaminated solid radioactive waste", stored mostly at Windscale and Drigg in Cumbria, are well within International Atomic Energy Agency safety limits. They do, however, exceed the dumping levels arranged by the Nuclear Energy Agency of the Organisation for Economic Cooperation and Development, and Britain is to push for higher limits.

SOME members of the Council of the Royal Society for the Prevention of Cruelty to Animals (RSPCA), following their success in persuading the society to oppose fox hunting, are trying to engineer the acceptance of a similar policy towards angling. They say it is cruel and should be banned. If they are successful, the conservation of our environment, and in particular of our rivers, could suffer great damage.

Angling is by far the most popular outdoor sport in Britain, having as many as three million adherents. On an average weekend during the fishing season, there are likely to be as many as five individuals sitting patiently by our rivers and canals waiting for a fish to bite for every one spectator attending a professional football match. Although some members of angling clubs from the midlands may lighten the tedium of their coach journey home from the dykes of East Anglia by song lubricated with bottled beer, this seldom if ever leads to the sort of vandalism apparently inseparable from watching some of our more notorious soccer teams. The unobtrusive behaviour of fishermen generally is the reason why others are so surprised to learn of their numbers.

However, anglers can be aroused to effective action when their waters are polluted. Although salmon and trout can flourish only in the cleanest streams, even so-called "coarse" fish (which supply the bulk of the sport in England) are delicate indicators of pollution. There have been thousands of occasions when the first warning that some unauthorised and toxic effluent has been discharged into a river has been given by a fisherman who has spotted dead or dying fish which might never have been noticed by the river authorities. The Anglers' Cooperative has been effective in taking legal action to stop such practices. Of course anglers can only operate in

rivers pure enough to contain fish, and so our filthiest waters do not receive their attention. This is perhaps one reason for the slowness with which they are cleaned up. But once a river is clean enough to be invaded by fish, it is the angler who plays the

Angling right



KENNETH MELLANBY

greatest part in safeguarding it and who may be responsible for further improvements.

Anglers not only outnumber other sportsmen; they also greatly outnumber all our organised environmental and wild-life conservationists. Angling is, furthermore, a sport with adherents from all social classes, including those unlikely to be involved in the more elitist forms of conservation. Yet, as I have indicated, anglers are really *effective* conservationists. It would thus be to their mutual benefit if the two groups could get together to cooperate whenever possible. If bodies like County Conservation and Naturalists' Trusts could recruit a

proportion of the anglers operating in their area, they could often quadruple their numbers and end the criticism that they are only a bunch of middle class protectionists working for their own selfish ends. The conservation bodies could help the anglers, for instance, by making waters in suitable reserves available for fishing, and by preventing bodies like the RSPCA (many of whose members are the self-same middle class conservationists) from endorsing policies likely to damage the countryside.

Presumably the move to ban angling is an attempt to benefit the fish. It might prevent a number of fish from being hooked, an experience which can hardly be pleasant, though there is disagreement about the extent to which fish and other cold-blooded animals experience what we describe as "pain". However, without the vigilance of the anglers, fish kills would almost certainly become commoner, and fish might be completely exterminated from many of our waters. It is not unreasonable to suggest that the experience of a fish, choking to death in unnecessarily deoxygenated streams, or writhing in apparent agony after exposure to poisoned effluents, could involve greater cruelty than that arising from normal fishing practices.

Today there is such pressure on our countryside and on its wildlife that all forces need to be mobilised to protect the environment. Freshwater is the habitat most endangered by pollution. It is to be hoped that those members of the RSPCA who are so strongly opposed to fishing will come to realise what would be the eventual consequence of their present policy. Fortunately they are more likely to destroy their own society than to stop a sport which has such widespread support from all sections of the community, and which contributes so much to preserving our environment.

correspondence

Alternative refrigerants

SIR,—We have carried out a survey of possible substitute refrigerants that could be used in the event that it becomes necessary to ban the use of refrigerant-12, CF_2Cl_2 . Very few compounds meet the requirements of being nontoxic and nonflammable, having long-term stability in a refrigeration system and a short residence time in the lower atmosphere. A good alternative to R-12 must have a normal boiling point near -30°C . We have considered all compounds known to boil between -45 and -15°C . The list includes approximately 80 compounds of which all but seven can be immediately ruled out as good alternatives. The remaining candidates include CF_3I (b.p. = -23°C), C_3F_8 (b.p. = -36.7°C), $c\text{-C}_3\text{F}_6$ (b.p. = -33°C), $\text{CF}_3\text{CH}_2\text{F}$ (b.p. = -26.5°C), $c\text{-C}_3\text{OF}_6$ (b.p. = -29 to -30°C), CF_3OCF_3 (b.p. = -23.3°C), and CHF_2Cl (b.p. = -40.8°C).

Of these compounds CF_3I may have inadequate long-term thermal stability, decomposing to the products C_3F_8 and I_2 . We are uncertain as to the flammability of $\text{CF}_3\text{CH}_2\text{F}$, and it would be very difficult to prepare this partially fluorinated hydrocarbon in good yield. Perfluoropropane, C_3F_8 , appears to be an ideal refrigerant. We are concerned, however, that this compound would have a much longer atmospheric residence time than R-12 and thus build up to be photolyzed at much longer wave-atmosphere. This would be undesirable since it is not possible to predict beforehand all possible environmental effects of a contaminant. Because of the strain energy in perfluorocyclopropane, $c\text{-C}_3\text{F}_6$, we expect that this compound to be photolysed at much longer wavelengths than C_3F_8 and thus have a shorter atmospheric residence time. Also, the boiling point of this compound is nearer to that of R-12 and for this reason would be a better substitute. The cyclic ether, perfluoropropylene oxide, and perfluoromethylethyl ether appear to be good substitutes. Presumably, these compounds would rapidly decompose once released into the atmosphere. We are concerned that these compounds may have anaesthetic properties and that they may lack long-term stability in the presence of trace amounts of water. Refrigerant-22, CHF_2Cl , is already used in a wide range of applications. However, its boiling point is much lower than that of R-12

so that its vapor pressure is higher, and thus requires much sturdier and therefore much more expensive refrigeration hardware.

Our civilisation can function without aerosol cans, but not without refrigeration. Except for R-22, any of the alternatives we have suggested would require five to ten years to be produced at the required level. Any decision to ban the use of R-12 should take into account these facts.

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Carbon dioxide reduction

SIR,—As is well-known, the oil crisis raises three distinct technical problems: overall shortage of fuel, shortage of suitable portable fuel (now normally hydrocarbons), and shortage of raw material for organic chemical industry.

Several possible solutions have been advanced for the overall fuel shortage and, if they prove adequate, this should cease to be a problem. If not, we are in a more difficult situation than most of us yet realise.

It has been proposed that the use of hydrogen, readily available with sufficient power, may be an answer to the second problem. For the third no long term answer has been advanced; the use of coal will provide a temporary answer after oil is exhausted, and coal can also be used to produce portable hydrocarbon fuel.

I have already suggested (*Nature*, **251**, 469) that it is worth looking again at the production of furfural from agricultural waste as a partial, but renewable, answer to the second and third problems.

I do not myself believe that the technology of the liquid hydrogen economy will prove practical on the wide scale, and it seems to me that only organic compounds, though not necessarily hydrocarbons, can provide an answer to the need for a portable fuel.

Thus an answer to both the second and third problems may need a large scale, renewable source of organic compounds. The only inexhaustible source of carbon is carbon dioxide. Given an adequate supply of hydrogen, it is possible to reduce carbon dioxide as is normally done with carbon monoxide in the Fischer Tropsch reaction. It is the purpose of this letter to draw attention to this possibility, and to suggest

that the time for basic investigation of this reaction is now, before the need arises.

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Science and industry

SIR,—The urgent need for 'Harnessing Science and Industry' in Israel (February 12, page 442) must also apply in the UK. Indeed, discussion is urgently required as to how better use might be made of British scientific innovation and talent, and your leading article (January 29) has done something in this direction by touching upon the possibilities of encouraging more scientists into industry.

But the problem is both deep-seated and urgent. There was a move recently to focus attention on this problem by establishing an exhibit (and perhaps a study centre) in buildings surviving from one of the earliest science-based industries in this country—that is, on the site of the historic Perkin and Sons dyeworks at Greenford Green, Middlesex, a mid-nineteenth century enterprise with an enviable record for turning the results of the latest scientific research into technological and commercial success. Ironically, this move failed in January of this year with the deliberate demolition of the buildings of interest due to a lack of appreciation of scientific and technological matters by businessmen and civil-service administrators.

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IQ tests and majority groups

SIR,—In common usage, and by statute for elections in the USA, "majority" is a number of excess of half the total. In a total with three or more subgroups, the largest is a "plurality". A plurality may also be, but need not be, a majority. I suggest that in Dr Harrington's very valuable study (December 25, page 708), since none of the populations contained a majority, the largest group in each population was a plurality. His results seem to indicate that the more numerous, or principal, groups are favoured by current testing procedures; a most important finding indeed.

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news and views

Social insects and the evolution of altruism

from John Krebs and Robert M. May

It is an astonishing fact that many professional biologists still do not understand the essence of Darwinian natural selection. Many people still think, and write, that adaptations evolve by natural selection because they are 'good for the species as a whole'. This sort of statement can be found in such influential writings as Konrad Lorenz 'On Aggression' and the Nuffield Sixth Form Teachers' Guide. Natural selection is a matter of differential survival and reproduction of individuals (or to be precise of genes), not of species. Adaptations evolve because they enable genes in individuals to out-do genes in their conspecifics, and natural selection relentlessly pursues this course without regard to the survival of the species or population as a whole. For many purposes it does not matter whether one appreciates this distinction, but in analysing the evolution of social behaviour it is crucial, so it is not surprising that some of the most important developments in understanding natural selection have come from this field.

The central theoretical issue in the evolution of social behaviour is the problem of altruism. In biological terms, an altruistic act is one which decreases the fitness (survival and reproduction) of the actor, while increasing the fitness of another individual (the recipient). If such genetically altruistic behaviour occurred in nature, it would undermine the very core of Darwinian natural selection, which is why the problem is so interesting.

Charles Darwin himself remarked in the *Origin of Species* that the apparent altruism demonstrated by the social insects posed 'the one special difficulty which at first appeared to me insuperable, and actually fatal to my whole theory'. He then went on to give reasons why natural selection may sometimes operate at the level of family groups rather than individuals, in effect anticipating the idea of 'kin selection' which plays such an important part in modern ideas of the evolution of social behaviour in

animals.

Some types of apparent altruism such as parental behaviour are easily understood. A parent bestows care on its young, often at considerable cost to itself, but of course the young represent the parents' gene contribution to the next generation and natural selection by definition favours individuals who maximise their gene contribution to future generations. But children are not the only bearers of a parent's genes: brothers, sisters, cousins and so on all share a considerable proportion of their genes by common descent. So the same reasoning that applies to parents and their children applies to all kin. W. D. Hamilton, in two seminal papers (*J. theor. Biol.*, 7, 1, 17; 1964), developed the concept of inclusive fitness—the idea that in order to estimate the fitness of an individual it is necessary to take into account the survival and reproduction of kin. In practical terms, you might make a genetic profit by helping your brother to have an extra child, even at some cost to yourself, because your brother's child shares a quarter of your genes by direct descent. The general conditions favouring this type of 'kin altruism' can be stated precisely.

Your kin share a certain proportion of your genes, for example full siblings have a probability of 0.5 of sharing any particular gene by descent; nephews and uncles 0.25, and first cousins 0.125. This probability of sharing genes is referred to as the coefficient of relationship r ; more formally it is defined as the conditional probability that individual B has a particular gene given that individual A has it. An altruistic act will benefit (in genetic terms) the actor as long as the benefit to the recipient is greater than the cost to the actor by a factor equal to $1/r$.

In the case of full siblings if you sacrifice your life for your siblings, more than two must survive as a result of your death for the 'altruistic' sacrifice to increase your fitness. Any apparently altruistic behaviour which can be accounted for within this framework is genetically selfish and there-

fore consistent with Darwinian theory, for since gene types are the basic stuff of evolution, it is inclusive fitness, not individual fitness that is maximised by natural selection.

This important concept of inclusive fitness was used by Hamilton to explain the evolution of the most extreme altruistic behaviour in the animal kingdom, that of worker castes in the social Hymenoptera (bees, wasps and ants). In these social insects the reproductively competent queen produces sterile worker females, whose task is to spend their life rearing the other fertile male and female offspring of the queen. Worker behaviour superficially appears the ultimate in disadvantageous behaviour from the point of view of the individual perpetuating its genes. But, because in the Hymenoptera the males are haploid and the females diploid, the worker female is in fact more closely related to her sisters than to her own hypothetical offspring. Daughters contain a full set of their father's genes, and so sisters related through both parents have a higher degree of relationship to each other ($r=0.75$) than to their own children ($r=0.5$). Conversely, a male is more closely related to his daughter ($r=1$) than to his siblings ($r=0.5$). Females are related to their brothers with $r=0.25$.

So the daughters of a queen who are faced with the choice of rearing their own daughters or staying at home to rear younger sisters should prefer the latter course (which is possible in the social Hymenoptera because the generations overlap) because by rearing sisters, a female produces more of her genes in the next generation than by rearing the same number of daughters.

The haploid/diploid separation of the sexes in the Hymenoptera explains why sterile worker castes have evolved at least eleven times in this one group but only once (in the termites) in the whole of the rest of the class Insecta, in which both sexes are diploid, although the Hymenoptera comprise only 6% of all insects. A particularly

telling point is that this altruism is sex-limited: only females are workers and all males are reproductive.

In an exciting new paper, Trivers and Hare (*Science*, **191**, 249; 1976) have developed Hamilton's theory further and produced dramatic evidence to support its predictions. They begin by remarking that although this asymmetry in the way Hymenopteran individuals are related to one another predisposes daughters towards the evolution of eusocial behaviour (defined as that combination of cooperative brood care, division of the group into reproductive and sterile castes, and overlap of at least two generations, which characterises the 'truly' social insects), it does not compel it. Trivers and Hare have now clarified the precise circumstances in which asymmetric degrees of relatedness can lead to the evolution of eusociality and have generated several predictions which can be tested quantitatively.

Whereas a female worker is more closely related to her sisters than to her hypothetical daughters, this is not so for her brothers, with whom she has a r of only 0.25 (as Hamilton well realised). If a worker invested an equal amount of effort into rearing brothers and sisters her average r to the siblings she rears would be 0.5, no better than she would do by having her own children. Hence, if the workers are to make a genetic profit from their apparently selfless devotion to rearing siblings, they must put more effort into rearing reproductive sisters than into brothers. The appropriate extra effort follows from the r values above and is a 3:1 preference for sisters over brothers in the sexual brood (the ratio measured, for example, in dry weight). With this ratio, the gene which the worker shares with the male is three times as likely to be present at the foundation of the new colony than the gene which the worker shares with the female, thus cancelling out the initial difference in r between the worker and the male.

On the other hand the queen of the colony, who actually lays the eggs prefers an equal investment in the sexes among her progeny (as proved by R. A. Fisher many years ago as a general rule for all sexually reproducing organisms). So within the colony there is a conflict of interests: the apparently altruistic worker can only pursue the genetically selfish course dictated by natural selection if she devotes three times as much effort to reproductive sisters as to brothers, whereas the queen can only maximise her fitness by equal investment.

Therefore, when a mother queen lays all the eggs and the worker caste is completely in control of rearing the

larvae, the ratio of investment should approximate 3:1. On the other hand, if the queen has control over the ratio of investment, it should remain at 1:1. When one or more workers lay eggs the situation is complicated and intermediate. In no circumstances is there any advantage to altruistic behaviour by the haploid males.

Trivers and Hare present good evidence, culled from various sources, for the sex ratios of reproductives ('alates') produced by some 21 ant species in which one queen lays all the eggs (monogynous). Sex ratios and relative weights are obtained by digging up complete nests during the time when alates are present, and from these data an estimate of the relative cost to a colony of a female alate compared to a male can be made. There is a good fit to the predicted 3:1 ratio; the departure from a 1:1 ratio is highly significant ($P < 0.01$).

Various other tests are carried out, although in most cases the data are softer or scarcer, and the points on the regression plots more scattered, than for the monogynous ants. Thus in slave-making ants, the queen's brood is reared not by her own daughters but by slaves (stolen from other species) who have no genetic relationship to the brood. Consequently the queen should achieve her preferred ratio of investment of 1:1. Two slave-making species indeed have ratios close to 1:1, while three closely related non-slave makers (of the genus *Leptothorax*) adhere to the 3:1 ratio. In polygynous ant species, a queen may permit one or more of her fertilised daughters to settle within her nest: large polygynous nests may contain granddaughter or even later generation queens. This makes for a complicated situation, with a tendency for ratios of investment to be biased towards males. Such seems to be the case for seven polygynous species for which data are available. The Hymenoptera also include solitary, non-social bees and wasps. Estimates of the ratios of investment in twelve species of

solitary bees and five species of solitary wasps are scattered near 1:1, and none are greater than 2:1. Corresponding ratios for eusocial bees and wasps are hard to come by, because there are time-consuming technical difficulties in distinguishing reproductives from workers. The ratios of investment for five species of social bumblebees (*Bombus*) are scattered in the range 1.2:1 to 3:1.

Trivers and Hare's paper is of profound importance. It puts the capstone to an edifice that draws together genetic principles and Darwin's theory of natural selection towards explaining the evolution of the social insects. Many other questions now open up. For example, among the most complicated caste structures are those belonging to some of the 'leaf-cutter' ant species of the tribe Attini. These ants bring plant material into their nests, where it is used to culture fungi which they eat: very crudely, they may be thought to have accomplished the transition from the 'hunter-gatherer' existence of most social insects, to an 'agricultural' society. It is surely not coincidental that they possess one of the richest caste systems, and it will be interesting to understand how it came about.

Besides providing direct evidence for Hamilton's kingship theory, Trivers' data are the first to test the outcome of parent-offspring conflict. This type of conflict must be widespread in animals with parental care, because a child is related to itself by a r of 1.0 and to its mother by 0.5, so that the two individuals have different genetic interests dictating their behaviour. Although R. D. Alexander (*Ann. Rev. Ecol. Syst.*, **5**) has developed the hypothesis that parents always have the upper hand over their offspring, Trivers' data show the opposite.

As so often happens in the evolution of ideas, it now seems that the social insects, which initially seemed to threaten Darwin's theory, are providing it with one of its most dramatic and quantitative triumphs. □

Organometallic chemistry

from Ronald Mason

'WHAT is the use of a book' thought Alice 'without pictures or conversations'. The hundredth issue of the *Journal of Organometallic Chemistry*—a growth stock if ever there were one—is a unique issue in that the editors invited some of the leading research workers to contribute a personal account of their involvement in the field and discuss its development. The result is fascinating—the single picture

is of lamentable quality but the conversations go a long way to eroding the old adage about scientists not understanding history (and *vice versa*!).

Organotransition metal chemistry owes much of its present shape and status to the discovery of the π -cyclopentadienyl and *bis*-benzene 'sandwich' compounds in 1951 and the two Nobel Laureates associated with the discovery—Fischer and Wilkinson—provide

articles which are interesting in their contrast. Wilkinson's recollections are intensely personal and warming to any advocate of the proposition that science is, above all else, a human activity. Fischer chose to discuss his most recent results on carbyne-complexes so we are deprived of the comparative viewpoint. Retrospectively, one of the more interesting points about the immense literature which has been generated by the original work is that the compounds have provided little direct spin off, most of the present concern in organometallic and low valent complex chemistry concentrating elsewhere, with metal carbonyls, π -alkyl complexes, tertiary-phosphine and -phosphite complexes and σ -bonded compounds providing more scope in synthesis and catalysis; if one accepts this argument, the articles provide for introspection and the prospects for the subject are not easily discernible.

The future for organometallic chemistry, and indeed for chemistry, is stiffly linked to the development of new approaches in synthesis. The synthetic strategies of organometallic chemistry are much less determined and sophisticated than those of organic chemistry but the serendipities of the past twenty years have been signposted and a rational basis for synthesis is emerging. Thus, so far as σ -bonded transition metal organometallics are concerned, the realisation of the difference between their kinetics and thermodynamic stabilities has led several research groups to design stable molecules through precluding, *inter alia*, the β -elimination reaction. The use of metal atom vapours in synthesis is providing species which had wrongly been thought of as thermodynamically unstable and these complexes may see much use as precursor reagents. The philosophy of the stabilisation of reactive species such as cyclobutadiene and benzyne is encapsulated in an article by Pettit and extrapolated to metal-carbenes and -carbynes which have much potential as intermediates in synthesis. In the context of the use of metals in organic synthesis, an obvious omission is one which takes no explicit account of the work of Wilke and his colleagues, particularly the recent studies in chiral and heterocyclic ring synthesis.

It will be interesting to see whether, as I believe, there will be a substantial shift soon in emphasis from organo-transition metal chemistry to main group organometallics. Brown, Gilman and, to a lesser extent, Hawthorne help us to anticipate the move but the main reasons for such a change are that following a lengthy period of establishing the basic methods of synthesis and general properties of Group IVB-organometallics, many novel advances

are coming along quickly including the fairly convincing demonstration of the existence of long-sought species such as $\text{Me}_2\text{Si}=\text{CH}_2$ and $\text{Me}_2\text{Si}=\text{O}$, the easy generation of silylene species, and the discovery of a wide range of novel rearrangement reactions. The upsurge of the use of organosilicon compounds in organic synthesis has reached into industrial processes with trimethylsilyl compounds involved in the manufacture of antibiotics. Eaborn touches lightly on this area in the hundredth issue, particularly with respect to the potential synthetic use of aryl-MR₃ bond cleavage. And one can remember the long standing use of organo-lead and organo-tin compounds. A long term future for main group organometallics is assured again by the recent discovery of the biological activity of certain cyclic siloxanes, which have similar properties to the oestrogenic steroids, and of the silatranes whose versatile therapeutic properties include claims for the treatment of baldness! One might go further, parenthetically, and generalise beyond organometallic chemistry: main group chemistry is now ready for the intensive approaches and techniques which have given a maturity to 'd' block chemistry.

It seems a pity that structural aspects of organometallic chemistry are hardly considered explicitly, given the symbiotic relationship of synthesis and, in particular, X-ray crystallography. Our understanding of the metal-carbon bond has changed profoundly, not least because of the insight which the Dewar-Chatt model provided for metal π -complexes. It is obviously a matter of personal taste and prejudice but, given his immensely varied and seminal contributions, I would have preferred to see Chatt discussing metal-carbon bonds and the reactivity of low-valent complexes, rather than highlighting the recent advances in metal-dinitrogen chemistry; but that part of his article which deals with the formation of nitrogen-carbon bonds can be assured of concentrated study over the next five years. Cotton's article on the dynamic or fluxional behaviour of organometallics in solution substantially remedies the balance but here one wonders if the last milestones have not been passed and that it will be other approaches which will provide a quantitative description of intramolecular potential energy surfaces.

A final thought is appropriate on the mechanisms of reactions of organometallic molecules, a topic which is in a primitive state compared with that obtaining in physical organic chemistry. Surely this is an area of mainstream development for it involves study of the reactions of coordinated ligands and the metal-activation of

covalent bonds, two matters which lie at the heart of any discussion of homogeneous and heterogeneous catalysis. Stone's contribution reminds us of the tremendous effort that has gone into studies of organometallic and coordination complexes of d⁸ and d¹⁰ metal ions and which have provided a variety of homogeneous catalysts and, for example, a much better understanding of the role of steric effects in reactivity (*vide* the activation of carbon-hydrogen bonds). The chemistry of polynuclear complexes has only just begun in anything like a systematic way and persuasive evidence is already available that some may not only be effective catalysts in their own right but their reactions can provide insight into the spectrum of physical and chemical processes that occur at surfaces of heterogeneous catalysts.

There have been sceptical, well-publicised comments on the maturity of organometallic chemistry which have carried the implication that the field will provide few novelties in the future. This proposition can be debated over all researches in the natural sciences but the prospects of metal ions providing more versatility and control over the reactions of organic molecules seem clearer and more rationally based than those of a decade ago. The two hundredth issue of the *Journal of Organometallic Chemistry* could provide an acid test of this forecast. □

Revolutionary SiC fibre

from Robert W. Cahn

G. K. CHESTERTON once wrote a rollicking poem about the Rolling English Road, and commented affectionately on the entertaining journey from somewhere-or-other to Birmingham, by way of Beachy Head. Anyone who knows England knows that it is not sensible to go to Beachy Head if you want to finish up in Birmingham, no matter where you start from. But it was an Englishman who had the crazy notion of making pure carbon (Birmingham) by first making an elaborate polymer (Beachy Head) and then destroying it by heat. Watt's technique of stretching oxidising and pyrolysing polyacrylonitrile to create strong carbon fibre was highly unconventional, but it worked triumphantly.

Now an equally eccentric and equally brilliant Japanese chemist, Seishi Yajima, has found an equally unconventional way to make strong, continuous fibres of silicon carbide. A preliminary summary of his method has been published in *Chemistry Letters* (Japan), (No. 9, 931; 1975).

More detailed publication is on the way, and Yajima (who is head of the Oarai branch of the Research Institute for Iron, Steel and Other Metals of Tohoku University) has also privately circulated an account of his underlying philosophy. He explains that conventional organic chemists have long striven to convert inorganic materials to organic (this goes right back to Wöhler's synthesis of urea), but they have an inbuilt resistance to turning back an organic material, attained in complex form by considerable efforts, into an inorganic material of simple structure by heat or chemical treatment. 'The organic chemist intrinsically detests a high temperature. He cannot stand the destruction of a complicated structure which has been obtained by painstaking efforts.' Yajima is an unconventional organic chemist who has overcome the phobia of fire.

Fibre-reinforcement for service at high temperatures is fraught with difficulties. Carbon fibre is too reactive, alumina is too weak, SiC whiskers are too short and too expensive. Intrinsically, however, SiC has all the required properties, and Yajima set out to make strong, continuous fibre. He starts with dimethylchlorosilane, $(\text{CH}_3)_2\text{SiCl}_2$, reacts it with lithium and a catalyst to form dodecamethylcyclotrihexane, $[(\text{CH}_3)_2\text{Si}]_6$, which is polymerised in an autoclave. The molecular weight fractions are separated by repeated solvent extraction and a solution of molecular weight $\sim 1,500$ is solvent-spun into a fragile, brittle fibre. This is then pyrolysed in a vacuum at temperatures in the range $800\text{--}1,300^\circ\text{C}$ to turn it into β SiC. Infrared spectroscopy and X-ray diffraction were used (the details are in process of publication) to follow the stages of pyrolysis. The best fibres ($1,300^\circ\text{C}$ heat-treatment) have strengths of $300\text{--}350\text{ kg mm}^{-2}$, and elastic moduli of 30 mm^{-2} . Diameters are typically $10\text{--}20\text{ }\mu\text{m}$.

The extraordinary feature of the fibres is that although their strength matches that of SiC whiskers, their structure is totally different. Electron microscopy and analysis of X-ray diffraction line profiles both reveal a structure of minute crystallites, 7 nm across, without preferred orientation. This fine structure is crucial: if pyrolysis is done at $1,500^\circ\text{C}$, giving considerably larger crystallites, the strength falls sharply. Prolonged attempts were made to match this structure by sintering ultrafine SiC powders, but these were never fine enough to achieve high strength. Yajima can only speculate as to the reasons for the remarkable strength associated with the ultrafine morphology.

The SiC fibres are extremely resistant to heat and oxidation, and will withstand, undamaged, prolonged exposure under load to a flame at $1,200^\circ\text{C}$. By contrast, SiC whiskers lose strength above 600°C . No information is given about the economics of the process, but it appears that the process can be made to work on an industrial scale.

Yajima claims that his work is the first instance of a metal atom being introduced into the skeleton of an organic polymer. This innovation has resulted in an inorganic product which appears at last to open the way to an effective fibre-reinforced composite, SiC-in-metal, which can give effective service at high temperature. $\square$

Signals for protein synthesis

from a Correspondent

WHAT features of mRNA primary or secondary structure are responsible for protein synthesis? Possession of a 5'-terminal AUG allows synthetic polynucleotides to function in many initiation assays but initiation codons are by no means unique to initiation sites. In eukaryotic messages, 'cap structures' have recently been shown to aid ribosome binding (Both *et al.*, *Cell*, **6**, 185–195; 1975) but no comparable feature has been found within prokaryotic mRNAs. Here the picture appears to be more complex, and considerable perception on the part of Shine and Dalgarno (*Proc. natn. Acad. Sci. U.S.A.*, **71**, 1352–1346; 1974) was required to identify what, recent evidence suggests, is indeed a signal for bacterial ribosomes.

Shine and Dalgarno point to a group of residues, mostly purines, found preceding the initiation codon by about 10 bases in the various initiation sites which have been sequenced—this partially conserved sequence could potentially base-pair with the 3'-terminus of 16S rRNA. They cite evidence implicating this part of the rRNA molecule in initiation and postulate that the selection of an initiation site is influenced by the potential of any given site to base-pair with 16S rRNA (Shine and Dalgarno, *Nature* **254**, 34–38; 1975). What is lacking in this imaginative hypothesis is any direct evidence that this base-pairing actually takes place, and it is this point to which Steitz and Jakes (*Proc. natn. Acad. Sci. U.S.A.*, **72**, 4734–4738; 1975) have addressed themselves.

They make use of the ability of colicin E3 to cleave 16S rRNA 50 bases from its 3'-terminus. When *E. coli*

ribosomes were allowed to initiate on the ^{32}P -labelled A protein initiation site of R17 phage and were then treated with colicin E3, the detergent SDS released most of the counts as a complex between the labelled initiation site and the 3'-terminal fragment of 16S rRNA. The complex was detected on polyacrylamide gels migrating more slowly than either the free initiation site or the colicin fragment—without colicin, the counts were recovered at 16S on sucrose gradients. The complex is an RNA:RNA duplex since it can be formed by the isolated RNAs, though presumably the extent of its formation is then much lower. The crucial observation is that if the binding of mRNA to ribosomes is inhibited by aurin tricarboxylate, only small amounts of this complex can be detected. Since this inhibitor does not affect the annealing of RNA, the most reasonable conclusion to draw is that binding of ribosomes to the initiation site facilitates the 'Shine–Dalgarno' base-pairing. This approach cannot tell us whether this interaction is occurring during initiation or during the subsequent disruption of the ribosomes. Few people would deny, however, that these experiments give substantial support to the hypothesis of Shine and Dalgarno.

If we may accept that base-pairing between the R17 A protein initiation site and the 3' end of 16S rRNA takes place during initiation, we must examine whether this phenomenon is of more general significance. Steitz and Jakes describe an experiment in which ribosomes were allowed to bind to intact R17 before exposure to RNase: the three initiation regions were then isolated as ribosome-protected fragments. Although the A protein initiation site shows the greatest complementarity with 16S rRNA, it is the site least favoured by this ribosome binding/protection reaction. Other, stronger signals direct ribosomes preferentially to the replicase and coat protein initiation sites. There is evidence from ribosome reconstitution experiments suggesting that, in addition to 16S rRNA, the ribosomal protein S12 plays a role in recognising the other initiation sites (Held *et al.*, *Biochemistry*, **13**, 2115–2122; 1974). Whatever signals are recognised in the replicase and coat protein initiation regions, they appear to lie outside the region conventionally isolated as a ribosome-protected fragment because, of the isolated fragments, only the A protein initiation fragment can bind efficiently to ribosomes (Steitz, *Proc. natn. Acad. Sci. U.S.A.* **70**, 2605–2609; 1973).

This is not to say that other recognition signals do not lie within the ribosome-protected regions, only that

in the case of the R17 synthetase and coat protein genes, these are not sufficient by themselves to permit efficient ribosome binding. Other features shared by many ribosome-protected fragments have been noted by Pieczenik *et al.* (*J. molec. Biol.* **90**, 191–214; 1974) in their studies on ribosome-binding sites from phage f1. First, the sequence coding for the first few amino acids often contains termination triplets in the two unused reading frames. These might serve to abort peptide bond formation after an out of phase initiation. Any feature characteristic of an initiation site, however, might be utilised in the recognition process and indeed, a role for the 3' end of 16S rRNA in recognising termination triplets was postulated by Shine and Dalgarno (*Proc. natn. Acad. Sci. U.S.A.*, **71**, 1342–1346; 1974). The other feature of coding sequences within initiation sites is palindromic sequences, that is, sequences such as C-A-G-G-A-C which 'read' the same in both directions. It is difficult to view palindromes as recognition elements since it would seem that they can only be identified by those who can read. In the absence of any evidence, both the out of phase termination triplet and the palindromic sequences should be considered characteristics of initiation regions. The only features presently identifiable as recognition signals are the 'caps' in eukaryotic mRNAs and the sequences complementary to 16S rRNA in prokaryotic mRNAs.

If the Shine–Dalgarno base-pairing signal is an important feature of initiation sites, strong selective pressures would act against any change in the relevant rRNA sequence. This sequence, however, has diverged somewhat within bacterial species and, in eukaryotes, the 3' end of 18S rRNA bears no resemblance to that of 16S rRNA, although this sequence is apparently invariant within the eukaryotes. Whether or not a similar scheme exists in eukaryotes is debatable. Dasgupta *et al.* (*Nature*, **256**, 624–628; 1975) have determined the nucleotide sequence of the initiation region of brome mosaic virus RNA-4—the mRNA for BMV coat protein. The sequence exhibits a weak, four-base complementarity with the 3' end of 18S rRNA. More interesting is the manner in which this region was isolated. The BMV RNA was subjected to limited RNase digestion and the fragments so produced were bound to wheat germ ribosomes. The region used for sequence analysis was then isolated from the 80S ribosomes as the only bound fragment which was annealed to the rRNA—the coding properties of this fragment demonstrate that it is indeed the authentic initiation region of this mRNA.

A similar observation suggesting a functional role for mRNA: rRNA base-pairing comes from Kabat (*J. biol. Chem.*, **250**, 6085–6092; 1975). Kabat isolates rabbit globin mRNA complexed to rRNA and finds that this complex is more active in translation by RNase-treated ribosomes than is the free mRNA. It is tempting to speculate that the RNase treatment has removed an exposed region of the rRNA which functions in initiation site recognition and that the exogenous rRNA can substitute for this missing sequence. It should be noted, however, that both 18S and 28S rRNA show this potentiation activity. Further studies with eukaryotic mRNA species will be required before conclusions can be drawn with any confidence.

What then is an initiation site? In bacterial systems it can be a fairly extensive region possibly extending into coding sequences. Within this region there may exist a number of features of primary or secondary structure which serve to direct ribosome binding. These signals must be available to ribosomes and not masked either by mRNA secondary structure or by proteins bound to the message. This latter may be an important control point regulating the synthesis of individual proteins in situations where transcriptional control is not appropriate (as in the RNA phages or in early embryogenesis). It is likely that as more extensive sequence information becomes available we will come to recognise more of the features responsible for directing the binding of regulatory proteins and ribosomes. □

Transform faults to spreading offsets

from Peter J. Smith

The term 'transform fault' has become an integral part of the language of the Earth sciences. It was introduced by Wilson (*Nature*, **207**, 343; 1965) to describe a fault that terminates abruptly at points where its motion is 'transformed' into a movement of a quite different feature, and was first used specifically in connection with offset spreading oceanic ridges, where strike-slip motion is limited to the zone between the two ridge segments. But this is a purely phenomenological definition carrying no information about how such faults originate. And therein lies a problem which according to Vroman (*Tectonophysics*, **30**, T11; 1976) has led to a "mire of misunderstandings".

The difficulty is that the, albeit necessary, adoption of a descriptive definition has apparently encouraged

many workers to assume that all faults conforming with it have the same cause. Not that the cause has been established, even for the familiar transform faults in the ocean floor. Shear fault patterns on land are the result of brittle fracturing in an elastic solid; and it has therefore seemed reasonable to many to assume that the dense grid of 'shears' representing transform faults in the solid ocean floor is produced likewise. In Vroman's view, however, this is not necessarily the case. On the contrary, the lack of a conjugate set in the pattern of oceanic transforms suggests that these offsets were created in material having Newtonian properties and responding to the laws of laminar flow. The apparent paradox that transform faults are now found in solid crust is then to be resolved by assuming that the faults are inherited from a time before the Newtonian liquid's solidification.

To develop these ideas, Vroman refers back to the original laboratory experiments of Oldenburg and Brune (*Science*, **178**, 301; 1972) who showed that spreading axes, transform faults and their associated fracture zones could be generated in freezing paraffin wax. One of the important results obtained by Oldenburg and Brune was that whereas the liquid material upwelling along a regular spreading axis solidifies and enlarges each plate equally, accretion takes place differentially along an initially jagged axis in such a way as to smooth out the irregularities. According to Vroman's interpretation of the Oldenburg–Brune model, however, spreading axes also exhibit more prominent irregularities which are essentially everlasting.

These 'kinks' are thought to play an important part in the development of the spreading pattern. As liquid is carried away to the two accreting walls of the spreading axis, a flow pattern is induced. But the kinks in the walls exert a frictional force on the liquid and thus produce finite differences in flow velocity which perturb the accretion process. The lines connecting corresponding kinks become zones of weakness which may fracture with the slightest encouragement, such as might be given by small thermoelastic stresses during the final stages in the cooling of the liquid. In short, Vroman suggests that there is a 'rate of shear' between neighbouring stream tubes which produces transform faults parallel to the stream lines at the liquid surface. On the other hand, some transform faults are evidently not related to kinks. In such cases, shear may result not from irregularities in the spreading axis walls but more directly from irregularities (inhomogeneous viscosity, for example) in the intervening liquid itself.

Vroman as good as admits that his interpretation of the Oldenburg-Brune model may be contentious, but as further evidence, points to the Gulf of Aden where transform faults can be seen to connect corresponding kinks in the opposing shelf margins. Moreover, this real example adds a further dimension to the argument in that the kinks here are old and evidently preceded the onset of spreading; they presumably occur where the new spreading axis intersects an old fault system. To this extent, therefore, transform fault locations may be governed by the prespreading history of the area concerned.

But be that as it may, Vroman's analysis clearly applies to the transform faults of the ocean floor. At the same time, however, there are several transcurrent faults in solid continental rocks which, because they happen to terminate at structural features which 'absorb' their motion, must also be termed 'transform faults' according to Wilson's strict definition. In other words, Wilson's definition includes faults of two quite different origins; and it is this ambiguity which has led to the misunderstandings and even "evil consequences".

It would appear to Vroman therefore that the term 'transform fault' is no longer restrictive enough to be useful. The unique character of oceanic 'transform faults' lies not in their transforming properties but in their origin. For this reason, Vroman proposes that the term be abandoned in the oceanic context and replaced instead by the name 'spreading offset'. □

Progress with X rays

from Andrew Fabian and James Pringle

On January 27-29 about 200 astronomers and physicists assembled at the Massachusetts Institute of Technology for a meeting of the High Energy Astrophysics Division of the American Astronomical Society. The subject was X-Ray Astronomy.

THE morning sessions were taken up with invited contributions, several of which were genuine and useful reviews of the invited topics. Too many speakers, however, provided an unbalanced view of the field with particular emphasis on their own achievements. The content of the contributed papers reflected the attitude of the conference organisers to theoretical matters. Of more than 70 short contributions accepted by the organisers, only three were on theoretical topics. This meant that not only was theory

underrepresented (and possibly misrepresented), but also that a number of the supposedly observational contributions contained more than their advertised share of theoretical explanation, some of it of dubious quality. Most of the contributed papers were of high quality and several interesting new results were presented.

The body of data on the pulsing X-ray sources is increasing. Most of the periods are in the range of minutes, but R. Lucke (Naval Research Laboratory, Washington DC) reported that the X rays from the source in the small Magellanic cloud, SMC X-1, are pulsed with a period of 0.716 s. The pulsed fraction is about 25% between 1.6 and 7 keV, and possibly variable at lower energies, when data from a rocket flight and the Apollo spacecraft 1.5 yr later are compared. S. Rappaport (MIT) showed preliminary results on the changing period of the pulsed transient source A0535+26, although the observations are not complete or sufficiently extensive to be very useful. G. Fabbiano (Center for Astrophysics, Harvard University) reported the discovery of a glitch, giant by pulsar standards, in the pulse period of Cen X-3. The period changed by about two parts in 10^5 in less than a day. W. Forman (Center for Astrophysics) has analysed some of the later Uhuru data and 23 new weak galactic sources have been found. The 'extended low' in the 35-d cycle of Hercules X-1 contains a phase where pulsed emission is detected at about 30% of the level of the normal 'on-state'. C. Jones (Center for Astrophysics) stated that this feature has been detected every time that this phase has been observed.

Evidence is also growing that sporadic burst behaviour may be a common feature in the X-ray sky. R. D. Belian (Los Alamos Scientific Laboratory) reported that a number of bright X-ray flares of a few minutes duration had been seen by the two Vela 5 satellites, possibly all coming from a region in the constellation of Norma. Several speakers reported transient X-ray behaviour at high galactic latitudes, and G. Jernigan (MIT) presented data on the 10-s flares first seen by the ANS satellite (Grindlay *et al.*, *Int. astron. Union Circular* No. 2879, 1976), from the direction of the globular cluster NGC 6624. A sequence of nine flares has been seen, recurring with an average of 15,718 s, although there appears to be a jitter of up to 1,000 s. Several other flares have been observed near this region of the sky, but directional information excludes the globular cluster.

One striking feature of the meeting was the quality of the spectra obtained from the Ariel 5 satellite. L. Culhane

(Mullard Space Science Laboratory, University College London) showed that the spectral data now acquired from clusters of galaxies require more sophisticated models than the simple one-temperature thermal bremsstrahlung fits previously used. Indications of an emission line, possibly due to iron, in the spectrum of the Perseus cluster provided a foretaste of possible abundance analyses in such distant objects. P. J. N. Davison (Mullard Space Science Laboratory) and S. H. Pravdo (Goddard Space Flight Center, Greenbelt) showed clear evidence for iron line emission from the Cassiopeia A supernova remnant, as viewed by the Ariel 5 and OSO-8 satellites, respectively. Another glimpse of the future was provided by P. Gorenstein (Center for Astrophysics). He presented an X-ray picture of the Perseus cluster obtained with an imaging X-ray telescope from a rocket flight lasting a few minutes. The launch of a similar instrument on the satellite HEAO-B must revolutionise the present hazy knowledge of the X-ray sky. □

The brain of the "reeler" mouse

from Shin-Ho Chung

Recent anatomical studies on neurological mutant mice have provided fascinating insights into the sequence of events leading to the formation of the brain. Before making synaptic contacts with one another, young neurones migrate from an inner mitotic layer to their eventual destinations where they form laminar aggregates. Because a defective gene in the mutant mouse disrupts a certain stage of this complex and precisely ordered sequence of events, analysis of the affected brain gives insight into both the action of the mutant gene and the normal mechanisms controlling neurogenesis. In some mutants, the genetic disturbance is confined to a specific region of the brain (News and Views, *Nature*, **257**, 86; 1975), whereas in others, such as the "reeler", the consequences of the defective gene can be noted in a variety of different cortical structures.

The autosomal recessive "reeler" gene arose by spontaneous mutation, and the homozygous mutants have an unsteady gait suggestive of inebriation (Falconer, *J. Genet.*, **50**, 192; 1951). Histological examination has revealed that cellular organisation in the brain is grossly disrupted, especially in the cerebellum, the hippocampus and the neocortex (Meier and Hoag, *J. Neuro-pathol. exp. Neurol.*, **21**, 649; 1962; Hamburg, *Dev. Biol.*, **8**, 165; 1963;

Cragg, *Expl. Neurol.*, **49**, 858; 1975). Instead of forming tightly packed layers oriented uniformly towards the cortical surface, cells in the corresponding layers of the "reeler" are very loosely packed and randomly oriented. Caviness and Sidman (*J. comp. Neurol.*, **145**, 85; 1972; *ibid.*, **147**, 235; 1973) have noted in the "reeler" a second systematic error in that the loosely packed layers are inverted relative to their positions in the normal brain. In the olfactory cortex, for example, the larger pyramidal cells are below an outer zone of small polymorphic cells, which is the reverse of the order in the normal brain.

The failure to achieve the normal laminar aggregates in the mutant brain is not caused by an abnormality in the time of cell generation or the ability of cells to migrate in their normal sequence (Caviness, *J. comp. Neurol.*, **151**, 113; 1973; Devor *et al.*, *ibid.*, **164**, 471; 1975). Autoradiography has shown that each class of cells in the "reeler" undergoes final mitosis at the normal time during embryonic development, and the onset of migration into the cortical plate also proceeds normally. During the terminal phase of migration, however, the mutant gene misdirects morphogenetic events governing cell position. Normally, newly mitosed cells migrate outwards towards their final position, with successive layers being formed by younger cells migrating through previously laid-down strata of older cells. Thus, in the normal animal, the younger the cell the further it has to migrate to its final destination. In the "reeler", this process is reversed. Instead of stopping at the correct location, the first wave of migrating cells advances to the superficial layers and subsequently generated cells take up deeper positions.

In spite of this eversion of cell layers resulting from aberrant cell migration, subsequent developmental processes appear to proceed normally. Trajectories of the principal axons to their appropriate regions are sometimes altered to seek out remote abnormally positioned cells (Frost and Caviness, *Proc. A. Meeting Soc. Neurosci.*, **4**, 217; 1974). In other regions where the path of growing axons remains unaltered, displaced cells compensate for the misalignment by extending their dendrites further than they normally do, meandering tortuously until the correct input is found (Devor *et al.*, *op. cit.*; Bliss *et al.*, *Brain Res.*, in the press). When examined electrophysiologically, the patterns of cell to cell connectivity are remarkably like the normal, at least in the hippocampus (Bliss and Chung, *Nature*, **252**, 153; 1974). Thus the brain appears sometimes to make use

of compensatory mechanisms to establish functional integrity.

That a single gene mutation causes the same sort of cellular disarray in very different parts of the brain suggests that there is a developmental mechanism with a unique molecular basis that is common to them all. It has been widely conjectured that the interaction between migrating young neurones and their input axons from other parts of the brain is crucial for the normal ordering of neuronal networks. In many areas, these input axons reach their appropriate sites coincidentally with or in anticipation of the arrival of their target cells. Such a precisely synchronised afferent-efferent interaction may be at fault in the "reeler". For example, the axons may be delayed in arriving or the young cells themselves may fail to interact properly with their partners. Alternatively, migrating cells may lack a surface property which facilitates aggregation once they have reached their destination. This line of speculation leads to a biochemical search for a missing or altered protein on the cell surface. In this context, it is relevant that cortical cells cultured *in vitro* from "reeler" mice do not aggregate to form the laminar arrays characteristic of cells from normal littermates (DeLong and Sidman, *Dev. Biol.*, **22**, 584; 1970). The sequence of molecular events linking the "reeler" gene to the abnormal pattern of cell migration will undoubtedly be unravelled; and the elucidation of such a process will be a landmark in the understanding of how the brain is formed. □

Deformation within continental plates

from J. Sutton

Now that it is possible to measure the very small movements that occur within continental plates a variety of researches become feasible. As a group of papers read at the fifth international symposium on Recent Crustal Movements (*Tectonophysics*, **29**; 1975) shows, the relations between movement in the Rhineland and the regional stress field produced in Central Europe as a result of the movements in southern Europe and the opening of the Atlantic are now well established. Compressive stress acting on north-west and south-east lines produces slip at rates of 0.05–0.3 mm yr⁻¹ in the Rhineland valley. These movements, slow by comparison with those in California or New Zealand, which may be 500–1,000 times as great, are accompanied by shallow earthquakes which occasionally cause damage and

deaths as at Basel in 1356 when 300 persons were killed. Ahorner (*Tectonophysics*, **29**, 233; 1975) emphasised how small are the movements which explain the seismicity of the region. At greater depths, flow of rock is important and Illies (*Tectonophysics*, **29**, 251; 1975) described how the mantle has risen for 6 km into the crust below the Rhinegraben rift system. Illies suggested that subcrustal masses displaced by plate collision, induced volcanism and intraplate rifting north of the Alps.

In last week's issue of *Nature* (page 648) Coward discussed another aspect of intraplate tectonics, the possible influence of pre-existing structures on present day movements. He described structures from near Adelaide, an area like southern Germany in that it lies between a plate boundary, the Pacific margin, and an ancient stable area in West Australia. In Australia as in Europe, recent volcanics are virtually absent where very old rocks outcrop (western Australia, Sweden and Finland), but are sporadically distributed through parts of both continents which have been deformed since the Precambrian.

Coward described deformation within a well known belt of folded rocks east and north of Adelaide. He has determined the extent of deformation across a belt some 150 km wide where shearing about 500 Myr ago produced displacements which he estimates to amount to 150 km. Making comparisons with other belts of intensely deformed rock formed by shearing parallel to their length, Coward suggested that the South Australian belt might have extended at least 10 times the length of such displacement. Such a long structure might be expected to affect both upper mantle and crust.

Coward went on to make the point that the Adelaide region is a zone of recent seismic activity and referred to the work of Cleary and Simpson (*Nature*, **230**, 239; 1971), in which they considered the distribution of earthquakes within the Australian continent, and where they discussed three seismic zones within Australia that could be lined up with sections of the Antarctic Ridge where earthquakes occur along offsets. One of these zones which passes near Adelaide and which follows the Ordovician structures mentioned by Coward, continues, Cleary and Simpson suggested, into the most westerly of the offsets in the oceanic ridge recognised by Sykes (*J. Geophys. Res.*, **75**, 5041; 1970) off South-East Australia. The implication is that the present day seismicity follows lines of weakness established within rock deformed much earlier in the geological history of Australia.

Such a relationship may illustrate a

conclusion drawn by Watterson (*Nature*, **235**, 521; 1975), that large scale ductile simple shear belts in which deformation is usually accompanied by a reduction in grain size, may produce a permanent weakness in the crust. Watterson suggested, following work in West Greenland, that diffusion at grain boundaries plays an important role in ductile deformation, and the reduction in grain size with the consequent increase in such boundaries permanently reduces the strength of rock so deformed. The South Australia belt discussed by Coward might have been weakened in this way (also see Dunnet, *Phil. Trans. R. Soc., Lond.*, **A280**, 641; 1976). Coward suggested that the transform fault which offsets the Antarctic ridge south of Adelaide formed where the zone of ductile deformation was crossed in early Tertiary times by the spreading axis from which Australia and Antarctica were to separate. Cleary and Simpson suggested that the pattern of seismicity within Australia—essentially a belt in the south-west, another passing through Adelaide to the centre of the continent and on to the western coast and a third and more diffuse zone running parallel to the Pacific coast from Victoria to southern Queensland—could be accounted for by contrasts between the ancient shield to the west and a relatively “soft” layer beneath south-eastern Australia. They noted evidence suggesting a strongly developed low velocity layer and probably, therefore, a substantial zone of low strength in the upper mantle below south-eastern Australia. The observations of Coward and Watterson indicate that belts of ductile shear which probably extended down to the base of the crust could introduce a further weakness into the lithosphere which would be reflected in the distribution of earthquakes, and which might have influenced the many Tertiary volcanic centres of eastern Australia. □

Spectrin-like proteins

from Dennis Bray

A RESEARCH worker at the Pacific Research Center, Honolulu, tries to prepare tubulin from sea urchin eggs by a standard method and obtains an unexpected result. The extract obtained with glycerol and a chelating agent forms a gel on warming as usual, but this is made of filamentous actin instead of microtubules. The actin is combined with two other proteins, one of which has a molecular weight of 220,000 (Kane, *J. Cell Biol.*, **66**, 305; 1975). A second investigator, by a different ocean, isolates the acrosome of

an echinoderm sperm. This is composed of unpolymerised actin and a high molecular weight protein with a similar behaviour on SDS-acrylamide to spectrin—the major structural component of mammalian erythrocyte membranes. The author suggests that the two might be related and, in collaboration, shows that human spectrin and rabbit muscle actin can interact (Tilney, *J. Cell Biol.*, **63**, 349a; 1974; Tilney and Detmers, *J. Cell Biol.*, **66**, 508; 1975).

Within a year two other laboratories find that they too can obtain clear watery extracts which set when warmed. One is from *Acanthamoeba*, the other from rabbit lung macrophages. In both of these the gelled material comprises actin together with one or two other proteins. The purified high molecular weight protein from macrophages binds directly to F-actin and is compared with spectrin (Pollard, *Biophys. J.*, **15**, 124a; 1975; Hartwig and Stossel, *J. biol. Chem.*, **250**, 5659; 1975). A new high molecular weight protein is isolated from smooth muscle. It is a major component and present in kidney, lung, liver and brain, but not skeletal muscle. On immunological evidence it is associated with the fibrillar arrays within cultured cells. It is not myosin and the authors compare it to spectrin and the recently described actin-binding protein of rabbit macrophages (Wang *et al.*, *Proc. natn. Acad. Sci. U.S.A.*, **72**, 4483; 1975).

The casual reader of these papers, trying to keep up with the breathless pace of developments in cell motility, will find them intriguing in content and captivating in style. He will also, unless he is careful, come away with the wrong conclusion. For, although each is distinct and in essential details quite different, there is a strong underlying similarity in approach and interpretation (which has been emphasised in the gloss above). Although it is not explicitly said, there are broad hints from the *dramatis personae* that there is a moral. The audience is led to make its own conclusions, one version of which is that a high molecular weight protein, similar to spectrin, is present in many kinds of cell and influences the polymerisation of actin. But is this justified? Let us examine the evidence.

To begin with the new proteins are not necessarily the same. They are grouped together under the generic description of ‘high molecular weight’ but in a way this is misleading. It implies that there is some structure or function that always goes with a polypeptide of more than 200,000 daltons, and we do not know this to be true. There is no reason why the molecular weights of these proteins should not be as precisely compared as any other—

but so far this has not been done. At present they could vary by as much as 30,000 daltons: which as evidence of identity is not compelling. Other properties, such as solubility and actin-binding are not quantitative in the present context and, moreover, show differences as well as similarities. Second, these proteins are not spectrin. Of those that have been compared directly on SDS-acrylamide gels, two are clearly heavier and one overlaps in only one of its components. The other obvious test—that of immunological cross-reaction—has so far not been reported, although at least one of the laboratories involved must have all the necessary ingredients. In fact, less than a year ago they used an antibody to spectrin to demonstrate that this protein is absent from most tissues (Painter *et al.*, *Proc. natn. Acad. Sci. U.S.A.*, **72**, 1359; 1975). As to whether the proteins are ‘spectrin-like’—the question is as insubstantial as it sounds. Criteria which have been advanced in support of this aetherial quality are high molecular weight (see above), solubility in low ionic strength solutions and 80% ethanol, absence of ATPase activity and failure to form filaments in isotonic buffers. Finally, they are not necessarily involved in actin polymerisation, although it would be of great interest if they were. The possibility arises because all but one of the proteins is extracted together with actin which later polymerises, but the direct evidence, which can only come from tests with purified components, is so far lacking. Therefore, although these new and noteworthy phenomena are clearly related, it is too early to say in what way. In particular, a simple explanation of the kind given above is at present untenable. □



A hundred years ago

THE *Morgenblad* of Christiania states that a singular phenomenon was observed there after a recent violent storm. A number of worms were found crawling on the snow, and it was impossible to find the places from which they had issued, everything being frozen in the vicinity. Similar circumstances were reported from several places of Norway.

MANY of our readers, we are sure, will rejoice to hear that a movement is on foot in Germany to abolish the crabbed printed German alphabet and adopt Roman type. We sincerely wish the movement may lead to the desired result, and that it will extend to the still more vexatious written alphabet. from *Nature*, **13** (March 2), 357; 1876.

articles

Suppression of malignancy in human cells

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Fusion between malignant and normal human cells results in the suppression of malignancy as measured by tumour formation. Malignant-malignant hybrids retain their malignant character. The human cell system has advantages for the study of the control of malignancy.

BOVERI proposed that malignant expression may originate from chromosome imbalance¹ and there is little doubt that the transition from a normal to a malignant cell involves alteration of cellular regulation events, which presumably stem from changes in the genome. Such alterations could conceivably be induced by carcinogens or oncogenic viruses, or arise spontaneously as a result of somatic mutation. It is possible, therefore, that a normal cell becomes malignant on addition of genetic material, as with oncogenic viruses, or because a genetic regulating function becomes inactivated, or because of a combination of these events².

Genetic analysis of malignancy (defined here as the ability of cells to proliferate and produce tumours in appropriate hosts) has been facilitated by the ability to hybridise somatic cells from the same or different animal species. The work of Barski, Ephrussi and associates³⁻⁵, using hybrids between highly malignant and normal or low malignant mouse cells, suggested that malignancy was a dominant trait; that is, all hybrid cell populations produced tumour in suitable hosts. Later, however, Harris, Klein and associates⁶⁻¹² demonstrated convincingly that malignancy is suppressed in hybrids between malignant and non-malignant or low malignant cells and that malignancy behaves as a recessive trait in these conditions. This disparity in results was later recognised as being due to the loss of chromosomes from the respective hybrid populations⁶⁻¹³. Based on extensive chromosome analysis, Harris *et al.*⁷⁻¹² proposed that a specific chromosome(s) is involved in the suppression of malignancy, and that when this chromosome(s) is lost the malignant trait is re-expressed. The frequency of production of tumours by hybrids between malignant and normal cells was apparently related to the stability of the chromosomes of the hybrid population.

Most reported genetic analyses of malignancy have involved intraspecific cell fusion, using cells of rodents and other species. I now wish to report the suppression of malignancy in human-human cell hybrids and discuss some of the advantages of this system.

Parent and hybrid cells

The malignant cell populations used were two HeLa variants D98/AH-2 and HBU which lack detectable hypoxanthine guanine phosphoribosyl transferase and thymidine kinase activity, respectively^{14,15}. Actively proliferating or senescent diploid human fibroblasts were used as the normal parent populations. An inoculum of 1×10^5 cells of either HeLa parent produces tumours in 100% of inoculated animals,

whereas 5×10^7 normal fibroblasts produce no tumours (unpublished).

Fusion of cells was accomplished with inactivated Sendai virus according to Harris and Watkins¹⁶. The procedures used to select hybrids are outlined in Table 1. Briefly, malignant-malignant hybrids between the two HeLa parents were selected in HAT medium¹⁷. As the normal diploid population had no genetic markers we used three semi-selective techniques. (1) HeLa variants were fused with senescent fibroblasts which had lost their proliferative capacity and hybrids were selected in HAT medium. HeLa parent cells died in HAT and the senescent fibroblasts would not grow in any growth medium; thus only HeLa-fibroblast hybrids survived and replicated. (2) Since it could be argued that normal cells at the end of their proliferative lifespan have accumulated errors that interfere with their normal regulatory functions¹⁸, actively dividing fibroblasts were treated with iodoacetate. Cells treated with iodoacetate die unless rescued by fusion with untreated whole cells (in this case HeLa variants)¹⁹. Hybrids were again selected in HAT medium. (3) HeLa-normal fibroblast mixtures at a ratio of 10,000:1 were fused and hybrids were selected in HAT medium. This semi-selective procedure took advantage of the fact that human fibroblasts divide very slowly at low population densities. Rapidly dividing hybrid clones were detected readily and removed from the few contaminating fibroblasts.

The morphological characteristics of the mass populations of parent and hybrid cells are illustrated in Fig. 1. The malignant-malignant hybrid ESH6 (Fig. 1d) retained the epithelial-like morphology of the two parental HeLa variants (Fig. 1b and c). The only distinguishing features were increased cell size and larger nucleoli. All the malignant-normal hybrids had a morphology intermediate between that of the epithelial-like malignant parent and the fibroblastic normal parent (ESH5 only illustrated in Fig. 1e). This intermediate morphology has been retained in mass culture for several months and at least 60 population doublings. Clones were selected, however, which more closely resembled either the fibroblastic or epithelial-like parent (Fig. 1f).

In their organisational behaviour the malignant-normal

Table 1 Selection of human cell hybrids

Hybrid designation	Parent cells	Selective medium
ESH2	D98/AH-2 + senescent fibroblast	HAT*
ESH3	D98/AH-2 + iodoacetate-treated fibroblast	HAT
ESH5	D98/AH-2 + proliferating fibroblast	HAT
	10,000:1 ratio	HAT
ESH6	D98/AH-2 + HBU	HAT

* HAT medium is Eagle's basal medium supplemented with 10% foetal calf serum, 1×10^{-4} M hypoxanthine, 4×10^{-7} M aminopterin, and 1.6×10^{-6} M thymidine.

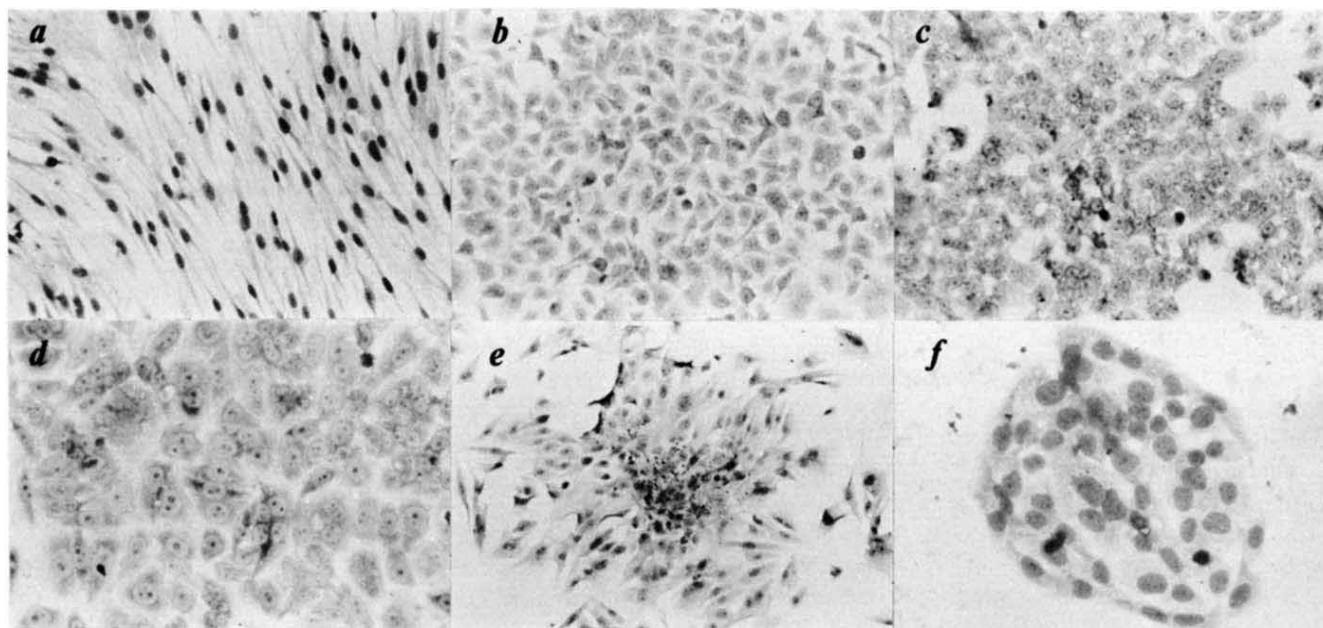


Fig. 1 Morphology of parent and hybrid cell populations. *a*, Normal fibroblast; *b*, malignant parent D98/AH-2; *c*, malignant parent HBU; *d*, malignant-malignant parent ESH6. The hybrid retains the epithelial-like morphology of parent cells shown in *b* and *c*. *e*, Malignant-normal hybrid ESH5. Morphology is intermediate between that of the parent cells shown in *a* and *b*. *f*, Clone of malignant-normal hybrid ESH3. Cells more closely resemble the malignant parent D98/AH-2.

hybrids more closely approximated that of the malignant parent cells. There seemed to be no density-dependent inhibition of division and the cells grew to high population densities (Fig. 2). In mass culture or in clones, however, the cells generally tended to be loosely packed and aligned themselves on their long parallel axes. Studies to be reported elsewhere have shown that the sensitivity of these hybrids to serum growth factors is intermediate between that of the malignant and normal parent cells. The malignant-malignant hybrids behaved in all respects like their malignant parent cells.

Chromosome constitution and assay for malignancy

Chromosome analyses of parental and hybrid cells are presented in Table 2. All the normal fibroblast cell strains used had a stable diploid karyotype. D98/AH-2 and HBU both had a relatively narrow modal distribution with means of 61 and 57, respectively (Fig. 3). The earliest time when various hybrids could be analysed was 4 weeks after fusion. In all cases, the mean chromosome constitution was slightly less than the expected mean calculated for the retention of

the total complement of chromosomes from both parents. Thus, as in other animal cell systems²⁰, there seemed to be early loss of chromosomes from hybrids. Whether the loss was random or non-random with respect to specific chromosomes has not been determined. After the early segregation events the chromosome content of the hybrids stabilised. We have observed very little loss of chromosomes in 5 months, during which the hybrid populations underwent at least 70 doublings (see, for example, ESH6 data in Table 2 and Fig. 4).

A problem when human cells are used for analysing malignancy is to find a suitable host in which to test the malignant potential of such cells. We have reported that human and animal malignant cell lines and biopsied human tumour material established and produced tumours in immunosuppressed mice, whereas normal cells failed to proliferate^{21,22}. We also found that when mixtures of malignant and normal cells were inoculated, only malignant cells proliferated to form a tumour whereas the normal cells failed to survive. We have purified malignant cell populations from mixtures of normal and malignant cells by this procedure²². We used three types of immunosuppressed

Table 2 Chromosome analysis of human parent cells and hybrid derivatives

Cell type	Expected*	Range	Observed	Expected*	Mean	Observed
Parent						
Normal fibroblasts	46		45-47	46		46
HeLa D98/AH-2	—		50-64	—		61
HeLa HBU	—		47-66	—		57
Malignant-normal hybrids						
ESH2 6 weeks after fusion	96-110		90-107	107		101
ESH3 4 weeks after fusion	96-110		94-106	107		104
ESH5 4 weeks after fusion	96-110		95-105	107		102
Malignant-malignant hybrid ESH6						
4 weeks after fusion	97-130		95-124	118		110
20 weeks after fusion	97-130		90-114	118		106
ESH6T 20 weeks after fusion†	97-130		95-118	118		107

Counts were based on at least 30 metaphase spreads.

* Estimated range and mean assume the presence of a full complement of chromosomes from the respective parent cells.

† Includes a total of 13 weeks in the mouse followed by re-establishment of tumour cells *in vitro*.

mice in the study reported here, all of which gave comparable results. The mice were (1) thymectomised neonatally and treated with antithymocyte serum; (2) weanlings that had been thymectomised, whole-body irradiated and reconstituted within 2 d with bone marrow or foetal liver cells²³; and (3) nude mice that require no immunosuppressive treatment because they lack a functional thymus and cannot mount a significant cell-mediated response against foreign antigens²⁴.

The results of the malignancy assays are presented in Table 3. Clearly only the malignant-malignant hybrid ESH6 retained its malignant properties. The fact that comparable cell numbers of ESH6 and the parent HeLa variants formed tumours is an indication that there was no partial loss of malignancy in the ESH6 hybrid. This was further substantiated by comparing the karyotypes of cells re-established *in vitro* from ESH6 tumours and ESH6 cells grown only *in vitro* for the same time (Fig. 4). The karyological profiles in Fig. 3b and c are very similar, indicating that there was no selection *in vivo* for a malignant subpopulation of the total ESH6 mass culture.

The suppression of malignancy in the malignant-normal cell hybrids seemed to be complete; as many as 1×10^7 cells inoculated failed to produce a tumour. Periodic monitoring of these cell populations during 6 months has yet to reveal a single tumour. It should be emphasised that only mass cultures have been tested. We are examining the malignant traits of a series of clones of different morphologies derived from the malignant-normal hybrid populations.

Malignancy as a recessive trait

The studies reported here clearly demonstrate that malignancy behaves as a recessive trait in human cells, and support the findings of Harris *et al.* who demonstrated sup-

pression of malignancy in mouse cell hybrids. Because of the preliminary nature of this report, it is not possible to conclude whether suppression is related to a specific genetic defect in the malignant cell which is made good by complementation with a normal cell⁶ or to a balance of chromosomes carrying information for expression or suppression of malignancy as proposed by Sachs and others²⁵⁻²⁷.

Weiner *et al.*^{10,12} found that several different malignant mouse cell lines failed to complement when fused together. This startling finding seems to suggest that malignancy in different cancer cells is due to a defect at the same genetic locus. My studies reported here do not help to clarify this intriguing possibility because the two HeLa variants used for the malignant-malignant fusion presumably originated from the same cancerous clone.

Fig. 2 Growth curves of parent and hybrid cell populations. 2.5×10^5 cells were inoculated into T-25 flasks (Falcon). Triplicate samples were taken at each time and counted in a haemocytometer. ●, WI-38; □, D98/AH-2; ○, ESH5; ■, ESH3.

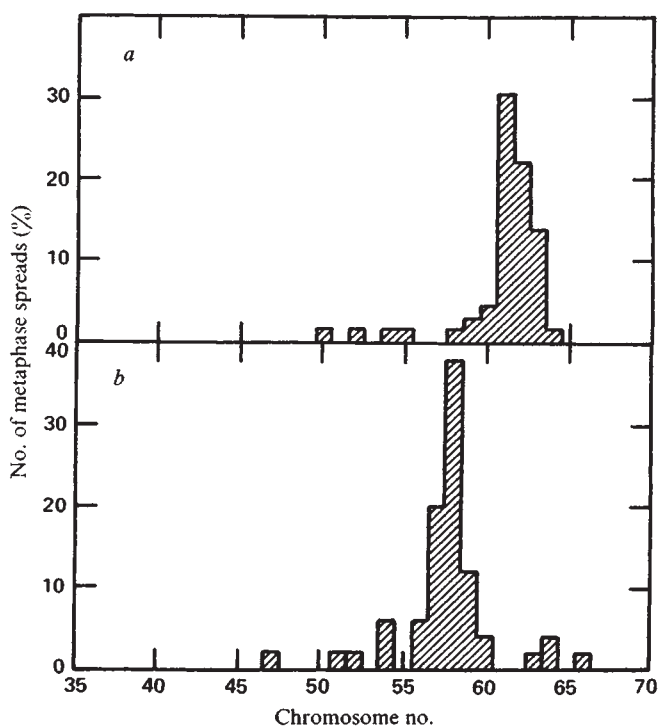
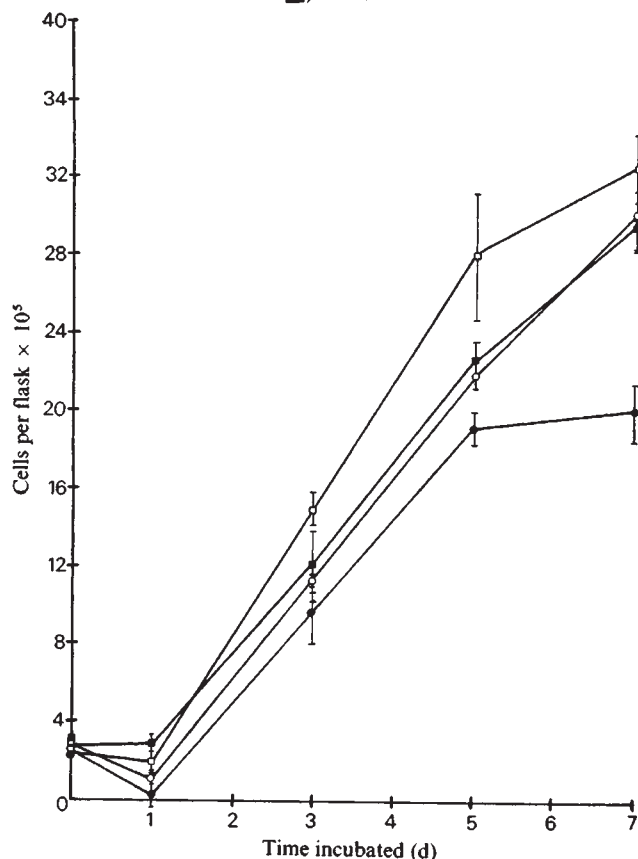


Fig. 3 Modal chromosome number of D98/AH-2 and HBU. a, D98/AH-2; b, HBU. At least 50 metaphase spreads were counted for each determination.

My results are at variance with those of Croce *et al.*²⁸ who reported that hybrids between SV40-transformed human cells and mouse macrophages retain the expression of SV40 tumour (T) antigen and are tumorigenic as long as the human C7 chromosome is retained in the hybrid. Loss of C7 was correlated with loss of T antigen and loss of tumorigenicity. Their findings indicate that the SV40-transformed phenotype is under positive control²⁹. The fundamental difference in our results may be explained on the basis that Croce *et al.* used human-mouse interspecific hybrids which may involve different regulatory factors. Also, the malignant human cells were transformed by an oncogenic DNA virus, SV40. The viral genetic information necessary for this transformation event may be able to supersede the regulatory control exerted by normal gene(s) which are necessary for the suppression of malignancy.

My studies with malignant-normal hybrids indicate that senescent normal fibroblasts can be rescued by fusion with malignant HeLa cells and attain immortality. Thus senescence of human fibroblasts in culture^{30,31} behaves as a recessive trait when fused with HeLa cells, a phenomenon

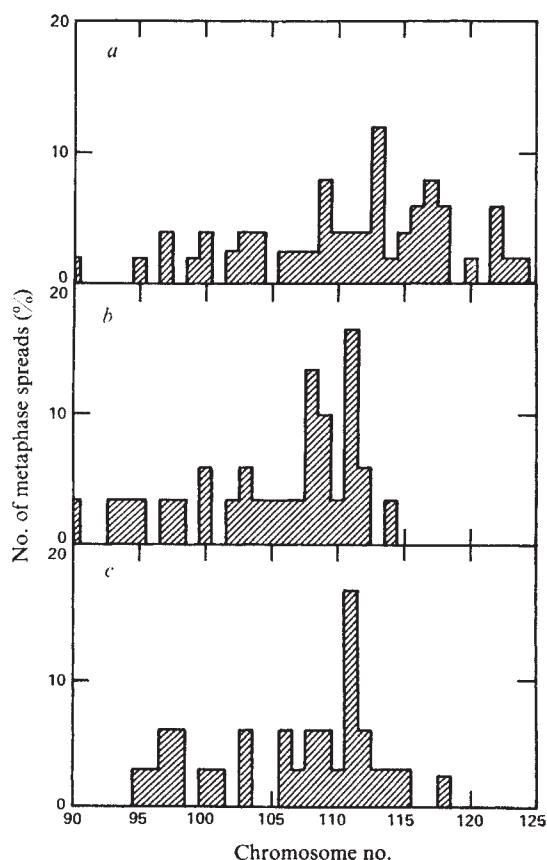


Fig. 4 Modal chromosome number of ESH6 at various times after selection. *a*, 4 weeks after fusion; *b*, 20 weeks after fusion; *c*, 20 weeks after fusion, including 13 weeks in a mouse. At least 50 metaphase spreads were counted for each determination.

noted by others^{32,33}. But although the malignant-normal hybrids can proliferate indefinitely, they are not malignant. The use of these human-human hybrids may provide a method whereby transformation events can be separated from the event(s) leading to malignancy.

The human-human hybrid cell system has several advantages over the murine or interspecific systems for the analysis of malignancy. (1) Most normal diploid mouse cell strains spontaneously transform into heteroploid malignant cell lines, thus normality is not a stable characteristic of mouse cells grown *in vitro*. Normal human fibroblasts have a stable diploid karyotype and a limited *in vitro* lifespan³¹, and have not been shown to transform spontaneously. (2) Many hybrids between malignant and normal mouse cells retain their malignancy when injected into animals⁷. This

apparent lack of suppression of malignancy has been shown to be due to rapid chromosome segregation from the hybrid cells. The rapidity of this chromosome loss, in addition to making the initial premise of suppression of malignancy hard to evaluate, renders the identification of specific chromosome(s) controlling this expression extremely arduous. My findings indicate that human cell hybrids have very stable chromosome constitutions which should render the isolation and characterisation of malignant variants easier. (3) Most, if not all, mouse cells contain endogenous RNA tumour virus genomes^{35,36}, but claims for the presence of human RNA tumour viruses in human cell lines^{37,38} have been unsubstantiated and probably represent contamination by viruses from other animal hosts³⁹. (4) Certain claims for the suppression of malignancy in mouse cell hybrids have been shown to be due to histoincompatibility rather than a genetic regulatory element⁴⁰. Because human cells are tested in a xenogeneic host, the question of histoincompatibility becomes a moot point since even malignant control cells would be rejected in inadequate immunosuppressive conditions.

Many other malignant-normal and malignant-malignant cell combinations must be tested before we can determine whether the suppression of malignancy reported here is a general phenomenon of human somatic cells. If this is the case, it should lead to significant advances in the study of control of malignant expression in humans. It remains to be seen whether non-malignant hybrids derived from cancer cells retain the antigenic identity of their malignant parent. If so, they may be useful in cancer immunotherapy. This possibility is strengthened by the fact that unlike mouse cells, human hybrids are chromosomally stable and seem to have their malignant phenotype completely suppressed.

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Table 3 Assay for malignancy of human parent and hybrid cell populations

Cell lines	No. of cells inoculated into each mouse	No. of tumours/No. inoculated Tx + ATS*	T-B**	Nude†
D98/AH-2	8 × 10 ⁵	5/6	10/10	6/6
HBU	8 × 10 ⁵	6/6	9/10	6/6
Normal fibroblast	1 × 10 ⁷	0/10	0/10	ND
ESH2	1 × 10 ⁷	0/10	ND	0/6
ESH3	1 × 10 ⁷	0/10	ND	0/6
ESH5	1 × 10 ⁷	0/10	ND	0/6
ESH6	8 × 10 ⁵	5/6	8/10	6/6

ND, Not done.

* Neonatal thymectomy plus antithymocyte serum.

† Thymectomy, whole-body irradiation followed by bone marrow reconstitution.

‡ Athymic nude mice, no immunosuppressive treatment.

Directed lipid flow in cell membranes

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A continuous and rapid oriented flow of lipid molecules in eukaryotic plasma membranes may explain capping of surface antigens.

MEMBRANES of eukaryotic cells exist in several discrete compartments¹. These include the rough and smooth endoplasmic reticula, Golgi apparatus, the nuclear and plasma membranes and various cytoplasmic vesicles. How these membranes are related to one another is unclear, but structures inside the cell must be responsible for maintaining the high degree of organisation observed in cell sections. Any cellular features or processes which display a unique polar property may tell something about the basis for this organisation. One such process is the capping phenomenon described by Taylor *et al.*² in lymphocytes and observed by several groups in other cells³⁻⁹.

Capping of antigens in the plasma membrane occurs when a particular surface antigen is cross linked, usually by specific antibody; the redistribution of these cross-linked antigens can be visualised using fluorescent antibodies. Two types of redistribution are found: the antigens form patches (which are just two-dimensional precipitates in the plane of the fluid membrane⁹) and are seen as discrete fluorescent areas on the cell surface. These patches may then form a cap, seen as a fluorescent crescent which in B lymphocytes occur at the end of the cell remote from the nucleus. Capping, unlike patching, requires metabolic energy and is thus an active process. In lymphocytes, where it has been most extensively studied, capping can be prevented by a combination of drugs which are believed to disrupt or inactivate microtubules and microfilaments—colchicine and cytochalasin B (ref. 10). Furthermore, once the cap has formed, addition of these drugs may enable it to disperse¹⁰. The main requirement, however, for molecules to be capped is that they be cross linked^{2,7,11}; other antigens, or antigens labelled with Fab antibody fragments, do not patch, nor do they cap.

The mechanism of cap formation has remained unclear, although it is generally believed that the surface antigens, once cross linked from outside the cell, are moved to one pole of the cell by cytoplasmic structures which may include microtubules and/or microfilaments¹²⁻¹⁵. Interpretations of capping along these lines become difficult to understand when one tries to consider them at a molecular level. How do the cytoplasmic components recognise that surface antigens are cross linked? Any simple explanation would seem to require that the surface antigens span the cell bilayer, a situation for which there are several precedents¹⁶⁻¹⁹. There then exist at least two plausible mechanisms for capping: either cytoplasmic components recognise a "new" distribution of those ends of the surface antigens on the cytoplasmic side of the membrane, and somehow select these to be capped. This mechanism is hard to contemplate as the membrane is a liquid and these surface antigens must frequently come close to each other due to Brownian motion. Alternatively, surface antigens could all be allosteric proteins; when cross linked from outside the cell, a conformational change of that part of the antigen on the cytoplasmic side of the membrane could be recognised as "new" by cytoplasmic components, and lead to the selective movement of these molecules to the pole of the cell. There are several difficulties with this hypothesis: (1) Fab fragments which bind to surface antigens might be expected to cause a conformational change—yet capping does

not result²; (2) apparently any surface antigen can be capped, which would imply a bewildering amount of recognition by cytoplasmic components; (3) lectins, which bind to sugar residues on surface glycoproteins, will cause capping of those molecules to which they bind^{7,11}, yet it seems unlikely that binding lectins to the carbohydrate portion of a protein would change its conformation.

Here I present a radically different interpretation of the capping process which may have interesting implications for other cellular processes.

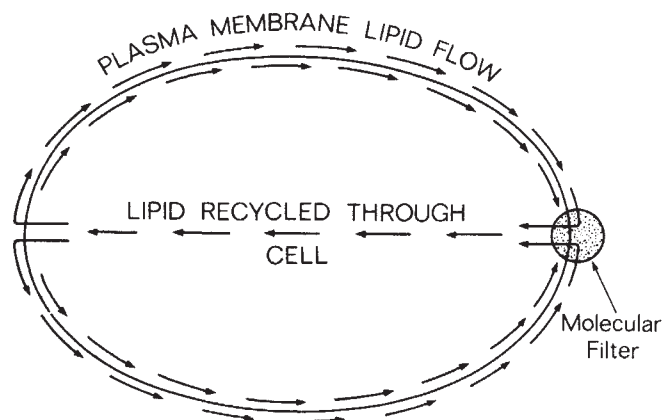
Continuous lipid flow in cell membranes

The hypothesis I wish to put forward is that there is a rapid, continual and oriented flow of lipid molecules in the plasma membranes of motile eukaryotic cells. Lipid molecules are inserted into the plasma membrane from the cytoplasm at certain sites on the cell and are resorbed back into the cytoplasm at different sites on the cell. This is shown schematically for a lymphocyte, in which there seems to be a lipid flow from one end of the cell to the other, in Fig. 1. Addition and removal of lipids presumably occurs through vesicles (see below). Other molecules associated with the plasma membrane do not cycle in this way: they remain approximately randomly distributed in the membrane under the influence of Brownian motion. The precise distribution of membrane proteins may not be quite random: the actual distribution will depend on many factors, including how fast the lipid flow is, the diffusion coefficient of the molecule in question and the length of the cell. But if a very large molecule is lodged in the plasma membrane, its rate of lateral diffusion may not overcome the lipid flow: it will then be swept to that site on the cell surface at which lipid resorption occurs. An example of such a large "molecule" is a set of cross-linked surface antigens. The individual antigens are no longer free to diffuse independently of one another and, if the patch is large enough, they will be carried along *en masse* by the lipid flow to the pole of the cell and will be seen as a cap.

Quantitative aspects of lipid flow

The idea that membranes flow is not new²⁰⁻²³. Ingram²⁴ and Abercrombie *et al.*²⁵ have observed the directed movement of large particles (such as carbon black) on the dorsal surface of fibroblasts from their ruffling edges to an area over their nuclei. This was interpreted as showing that the surface membrane of

Fig. 1 Schematic diagram of recycling of lipids in a lymphocyte.



these cells is flowing. The difference between their interpretation and my proposal is that I believe there is a net flow which is largely confined to lipid molecules—and not membrane recycling which includes all the membrane proteins. In the case of lymphocytes, it seems that once antigens have been capped, their reappearance on the cell surface takes a long time (compared with capping) and requires protein synthesis²⁶. In other words, capping does not seem to result from the recycling of surface membrane as a whole: if rapid membrane recycling exists, it must be limited to lipid molecules. Presumably the same holds for fibroblasts.

The rate of movement of particles on fibroblasts has been measured and is about $3 \mu\text{m min}^{-1}$ (refs 22 and 25). If a similar rate of lipid flow exists on a lymphocyte, then the time taken to sweep a cross-linked antigen on to one half of the cell (which is about $10 \mu\text{m}$ in diameter) would be about 2 min. This is roughly the time at which the first caps are seen to form on lymphocytes², and suggests that the rate of lipid flow in lymphocytes and fibroblasts is similar.

If a lipid flow rate of $3 \mu\text{m min}^{-1}$ exists across a flat lipid bilayer sheet, we can estimate how this will affect the distribution of individual protein molecules lodged in the membrane. The rate of lateral diffusion of rhodopsin molecules in frog retinal disk membranes has been measured by Poo and Cone²⁷: these proteins diffuse at a rate of about $10 \mu\text{m}$ in 1 min. If the viscosities in disk and plasma membranes are similar, the lateral diffusion of a protein the size of rhodopsin will effectively win out over a lipid flow of $3 \mu\text{m min}^{-1}$. There will, however, be a slight difference in concentration of antigens at the two ends of the stream. For a one-dimensional case in an equilibrium situation, the concentrations of a protein (C_a , C_b ; a is upstream of b) are given by $C_a/C_b = \exp(-FL/D)$, where F is the flow rate, L is the distance between points a and b in the direction of the stream and D is the diffusion coefficient of the protein. For a flow rate of $3 \mu\text{m min}^{-1}$, a diffusion coefficient of $4 \times 10^{-9} \text{ cm}^2 \text{ s}^{-1}$ (for rhodopsin in disk membranes at 20°C , ref. 27) and L of $10 \mu\text{m}$ (roughly the length of a lymphocyte), we get $C_b/C_a \sim 3$. Of course, the lymphocyte is a roundish cell so that F cannot be uniform over its surface and the parameters taken for rhodopsin may not be applicable here, especially as the viscosity in the plasma membrane of a cell growing at 37°C may be somewhat lower than in the frog retinal disk membrane²⁸. It is therefore difficult to predict just how great C_b/C_a would be. But if the protein molecule were made much larger, its diffusion coefficient (which is not easily estimated²⁹) would decrease and could thereby change C_b/C_a by a large factor because of the exponential relationship between D and C_b/C_a . In other words, as a protein aggregate grows in size, C_b/C_a may increase sufficiently that a patch would be seen to cap.

These calculations simply show that, given the rather crude assumptions made, a lipid flow of a few $\mu\text{m min}^{-1}$ could explain the capping process. They also indicate that surface antigens, or single proteins embedded in the bilayer, might not be quite uniformly distributed over a cell. This might be visible as a non-uniform distribution of particles in the freeze-fracture plane of a large cell, such as a fibroblast.

Implications of lipid flow

The most obvious prediction this model makes is that lipid molecules will not stay in the plasma membrane for long periods. More usefully, it predicts that a surface antigen need not communicate in any way with the cytoplasm to be swept along and thus be capped. Thus, phospholipids or glycolipids—molecules which by their nature only extend half way through the bilayer—should be able to be capped. The most direct way to test this would be to insert a new glycolipid into the bilayer of a cell not possessing that antigenic determinant, and see if that can be capped. This experiment cannot be unambiguous at present since there is no known way of showing that the added glycolipid is indeed properly inserted into the host cell's bilayer, rather than adhering to surface protein. Nevertheless, when such an ex-

periment is attempted³⁰, the added glycolipid does cap. A better experiment might be to see if antibodies which bind specifically to phospholipids³¹ could lead to capping.

There then remain two additional questions I should like to consider. If only lipids are resorbed into the cell, how is this done without concomitant resorption of plasma membrane proteins? A molecular filter would seem to be needed—a special vesicle designed to exclude membrane proteins. The other question is, where do the lipids go after internalisation? If they are fed into the internal membrane pool—the Golgi apparatus or endoplasmic reticulum—how is it that plasma membranes contain high levels of cholesterol (approaching equimolarity with phospholipids), whereas those membranes within the cell contain a low level of cholesterol which is only a fraction of that found in the plasma membrane³². In other words, the hypothetical filter I have in mind would remove not only plasma membrane proteins but might also remove much of the cholesterol. A possible candidate for this is the “coated vesicle”, which is found in many cell types^{33–39} and contains only one major protein, “clathrin”^{38,39}. These vesicles are believed to be responsible for resorption of plasma membrane in nerve endings after stimulation of the neuromuscular junction⁴⁰. They contain a small lipid vesicle inside a lattice network of clathrin^{38,39}; as the lipid vesicle buds inwards from the plasma membrane, clathrin can be seen to coat the vesicle^{33–35,39} and in so doing, might clear away other proteins to give a fairly pure lipid vesicle inside its coat.

Microtubules and microfilaments seem to be implicated in capping and thus in the recycling of phospholipids. How they do so is unclear, although there are hints that one of the functions of microtubules may be to shunt vesicles around^{41–44}, and microfilaments seem to be needed for various membrane movements such as ruffling^{45,46}. The present arguments imply that somehow these fibrillar elements are involved in the directed movement of membranes within the cell.

In conclusion the polar property displayed by many eukaryotic cells in the capping process or in the movement of particles adsorbed on their surfaces can be explained at the molecular level by an oriented and continuous flow of lipid molecules in the cells' plasma membranes. There must be a source and a sink for this lipid flow; the sink is likely to require a molecular filter which removes most of the membrane proteins and perhaps cholesterol from the internalised membrane. This specific resorption process may be achieved by coated vesicles.

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Jurassic igneous rocks of the Forties Field

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Middle Jurassic volcanic rocks unexpectedly encountered during North Sea drilling operations seem to have oceanic affinities. Though this finding may seem surprising in a suite situated in a continental basin, it can be explained using a plate tectonic model. According to the model, the volcanism developed along a line of incipient sub-crustal rifting during the early opening of the Atlantic. Before crustal splitting occurred, however, the spreading axis migrated westwards and the well documented volcanism of the North-west Tertiary Igneous Province developed.

DRILLING operations in the Forties and Piper areas of the North Sea have unexpectedly encountered basic igneous rocks which seem to be part of a thick volcanic sequence of Middle Jurassic age¹. As such, they represent a new and previously unsuspected igneous province in north-western Europe. The lavas, which have a maximum thickness of over 740 m, are porphyritic olivine basalts which "tend towards an alkaline affinity"¹. We report here on the whole-rock geochemistry of the lavas; our preliminary findings are based on material from the Forties Field wells 21/9–1 and 21/10–1.

The material from well 21/9–1 represents a 17-m vertical section comprising (in descending order):

- (1) The lowermost 0.65 m of a porphyritic basalt flow (the whole of which is probably not much thicker).
- (2) A 2.75-m thick flow of porphyritic vesicular basalt with a brecciated upper surface.
- (3) 12.6 m of porphyritic basalt which could be a single flow or as many as three separate flows.
- (4) The top metre of a porphyritic vesicular basalt flow of indeterminate thickness.

The section represented by the material from well 21/10–1 is comparatively short, comprising 1 m of porphyritic basalt on top of 0.5 m of porphyritic vesicular basalt, which in turn lies on top of sediments. The change from non- or sparsely vesicular basalt to markedly vesicular basalt may correspond to the junction between two flows, but the exact relationship is obscured by the altered nature of the rocks and the fact that just above the transition the porphyritic basalt is intruded by a 0.5-m thick sheet of vesicular 'dolerite' with chilled upper and lower margins.

Petrography

All of the flows represented in the well 21/9–1 section contain abundant, large phenocrysts of clinopyroxene (Cpx) and pseudomorphs after olivine (Ol). Phenocrysts of plagioclase are present in varying amounts but are much smaller. The pyroxene is virtually unaltered, but the olivine is almost entirely replaced by serpentine, chlorite and carbonate and often considerable amounts of iron oxide. The lavas from well 21/10–1 also contain phenocrysts of clinopyroxene and pseudomorphs

after olivine, but differ from those of well 21/9–1 in apparently lacking the plagioclase microphenocrysts and in containing abundant basaltic hornblende. The pyroxene phenocrysts in the lavas from both wells vary in size, but can be a few centimetres across, and exhibit spectacular, discontinuous, oscillatory and sectoral zoning. The mineral chemistry of the pyroxenes is still under investigation, but it is evident from their optical properties that they are augites with significant contents of Al and Ti, and are thus typical of the phenocrysts found in basic igneous rocks of alkaline affinity. This, together with the obviously high original olivine content, led Howitt *et al.*¹ to deduce that the rocks were probably alkali olivine basalts.

The groundmass of all the lavas is fine grained and very altered, but small plagioclase laths or microlites, minute augite crystals and magnetite/haematite grains can be discerned among the chlorite and other alteration products. As is typical of alkaline basaltic rocks^{2,3}, all of the specimens are more or less altered, particularly with regard to oxidation, which is most marked towards the tops of the flows. The top of the thickest flow in the well 21/9–1 section is so oxidised that it consists almost entirely of haematite. This red 'bole' is suggestive of subaerial weathering and seems to be incompatible with suggestions⁴ that the volcanics were subaqueous.

Although the indications are that the North Sea igneous rocks are of alkaline affinity it is difficult to be more precise on petrographic grounds, largely because of their altered nature, so we have investigated the whole-rock geochemistry. A number of samples from each of the flows represented in the two well sections have been analysed for major and trace elements (by a combination of X-ray fluorescence (XRF) and chemical methods) in order to study the systematic chemical variations which exist within and between individual flows (Table 1). More detailed geochemistry will be described elsewhere.

Whole-rock geochemistry

The classification of volcanic rocks on the basis of normative mineralogy becomes increasingly difficult and unsatisfactory as the rocks become more altered. Various methods have been suggested to overcome the effects of alteration, especially oxidation^{5–8}. We have followed Thompson *et al.*⁹ in reducing the Fe₂O₃ content to 1.5% or 2.0% (for total alkali contents of less than 4% and less than 7%, respectively) before calculating the norm. The representative analyses presented in Table 1 consist of the average compositions of two of the thickest flows from well 21/9–1 (9B, 9C), a typical specimen of lava from well 21/10–1 (11211) and the two samples from well 21/9–1 with the highest and lowest K₂O contents of those so far determined (3395.25, and 3396.5). All are nepheline normative except for the sample with the lowest K₂O content from well 21/9–1, which is only just hypersthene normative. Though this is consistent with alkali olivine basalt affinity, the altered state of the rocks does not permit unequivocal interpretation.

Another method of identifying the parentage of basic

Table 1 Analyses and CIPW norms of North Sea volcanic rocks

Number	9.C	9.B	11211	3395.25	3396.5
SiO ₂	44.61	43.96	44.27	44.20	43.59
TiO ₂	2.78	3.12	2.47	2.87	2.99
Al ₂ O ₃	12.84	13.95	9.72	12.51	13.39
Fe ₂ O ₃	6.91	7.13	4.44	6.09	8.85
FeO	4.45	4.53	5.84	5.43	3.46
MnO	0.22	0.22	0.20	0.40	0.14
MgO	10.60	9.18	14.27	11.26	8.66
CaO	9.84	8.40	13.01	12.13	7.07
Na ₂ O	2.36	2.61	1.48	1.75	3.13
K ₂ O	1.05	1.61	0.71	0.45	2.35
P ₂ O ₅	0.47	0.52	0.40	0.45	0.50
S		0.01	0.06		
H ₂ O ⁺	3.55	4.47	2.87	3.06	5.52
CO ₂	0.22	0.28	0.22		0.28
Total	99.90	99.99	99.96	100.60	99.93
Or	6.21	9.52	4.20	2.66	13.89
Ab	18.37	19.40	9.17	14.81	17.77
An	21.34	21.60	17.78	24.95	15.55
Cpx	Wo	10.19	6.97	17.85	13.48
	En	6.65	4.52	12.68	8.87
	Fs	2.84	1.97	3.62	3.66
Opx	En			0.08	
	Fs			0.03	
	Fo			0.09	
Ol	Fa	13.84	12.86	16.02	13.38
		6.52	6.18	5.04	6.09
		0.87	1.46	1.82	
Ne	0.87	1.46	1.82		
Mt	2.18	2.90	2.18	2.18	2.90
Il	5.28	5.93	4.69	5.45	5.68
Ap	1.09	1.21	0.93	1.05	1.16
Py		0.02	0.11		
Cc	0.50	0.64	0.50		0.64

volcanics is to plot them on a total alkalis against SiO₂ diagram (Fig. 1). It is evident that on this criterion the North Sea volcanics are, on the whole, mildly alkaline, with a few more strongly alkaline varieties.

The problems of correctly classifying altered volcanic rocks, especially alkaline types, has been discussed by a number of authors^{3,10,11}, particularly with regard to identifying their tectonic setting. Probably the most satisfactory method of typifying the North Sea basalts is the TiO₂ against Zr/P₂O₅ plot of Floyd and Winchester¹¹ which uses 'immobile' elements, thus minimising the effects of any subsequent alteration of the rocks. The North Sea lavas (Fig. 2) fall within the fields of both continental and oceanic alkali basalts and lie outside the fields of tholeiitic basalts. In addition, the suite exhibits the vertical trend characteristic¹¹ of alkali basalts and atypical of saturated volcanics. Trace element contents, particularly Sr and Rb are also consistent with alkali basalt parentage. The Sr and Rb contents are of the order of 600 p.p.m. and 20 p.p.m. respectively. Typical tholeiites contain less than 150 p.p.m. Sr and only a few p.p.m. Rb, whereas alkali basalts can contain up to 1,000 p.p.m. Sr and 100 p.p.m. Rb (refs 12 and 13). It therefore seems clear that the analysed Jurassic lavas from the Forties Field are mildly undersaturated rocks of alkali olivine basalt parentage.

Geochemical discrimination between alkali basalts from continental and oceanic tectonic settings is notoriously difficult (see Carmichael *et al.*¹⁸). One possible method is to use the TiO₂-K₂O-P₂O₅ ternary plot proposed by Pearce *et al.*¹⁴; but attempts to apply this method result in the analyses plotting along the boundary between the two types. Direct comparison of the North Sea lavas with other alkali basalts in terms of major elements reveals that individual analyses can be matched to a remarkable degree with basalts from such diverse tectonic settings as the East African Rift Valley^{15,16} and Hawaii¹⁷. Surprisingly, in view of the continental nature of the North Sea crust, the lavas seem to have more in common with oceanic alkali basalts than with those from continental settings. Indeed, in many geochemical respects, the alkali basalts from Hawaii seem to be the most directly comparable with those from the North Sea. With regard to this occurrence of alkali olivine basalts apparently having oceanic affinities within a continental

plate, it is worth considering the structure of the North Sea Basin at the time of the volcanism and its possible relationship to the large scale tectonic pattern.

Plate tectonic development

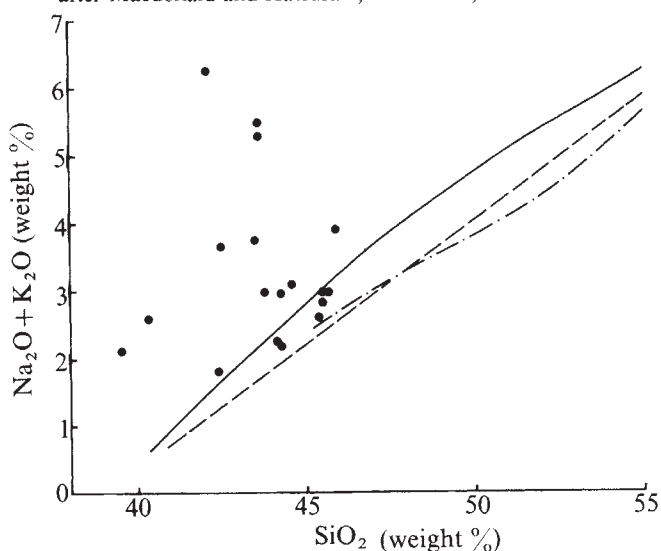
During the Jurassic the North Sea area was dominated by a series of major troughs, or graben. The volcanic rocks occur close to the confluence of three of these—the Witch Ground, Viking and Central Graben¹. It has been suggested¹⁸ that this trough system may have originated in Hercynian times but if so there seems little doubt that it was being reactivated during the Jurassic⁴. Tectonically, therefore, the best analogy for the Jurassic North Sea may be the East African Rift Valley where a comparable rift system is developing on continental crust, and the similarities between individual volcanic rocks of the two areas lends some support to this possibility.

The general north-south rifting in the North Sea area during the Jurassic could be connected with the subduction of the Tethys plate below the Russian Platform far to the south-east⁴, or, alternatively, it could simply be a result of the stress system developed at continental margins because of differences in crustal density¹⁹. Neither, however, could account for the thinning¹⁸ of the continental crust, or the occurrence of the igneous rocks, both of which are consistent with high heat flow in the underlying asthenosphere. Whiteman *et al.*¹⁸ have suggested that the North Sea rift systems may be failed arms of plume-generated rrr trilete junctions, but they attribute the rift system in the Forties area to uplift caused by mantle plume activity during the late Carboniferous and early Permian. Consequently, though mantle plume activity could certainly account for igneous activity, the timing is inconsistent with the Middle Jurassic age of the North Sea volcanic rocks and, in any case, there seems to be little evidence other than the rift systems to support the suggestion that plume activity occurred beneath the North Sea area from the Carboniferous to the Cainozoic.

We suggest the following model based on a general consideration of Laurasian global tectonics at the time of volcanism.

During the Lower Jurassic (approximately 180 Myr BP) the Atlantic began to open south of the Azores^{20,21}. The spreading axis is generally regarded as being truncated by a transform plate margin running from south of Spain to south of Newfoundland. By the beginning of the Cretaceous the mid-Atlantic spreading axis extended much further north, "possibly as far as the western approaches to the English Channel"²¹, and it seems reasonable to presume that this northward extension existed in an embryonic form during the Jurassic. When it matured into

Fig. 1 Total alkalis against SiO₂ plot for North Sea volcanic rocks. Lines denote the division between alkaline and sub-alkaline types: —, after Irvine and Baragar⁶; — · — · —, after Macdonald and Katsura¹⁷; — — — —, after Kuno⁸.



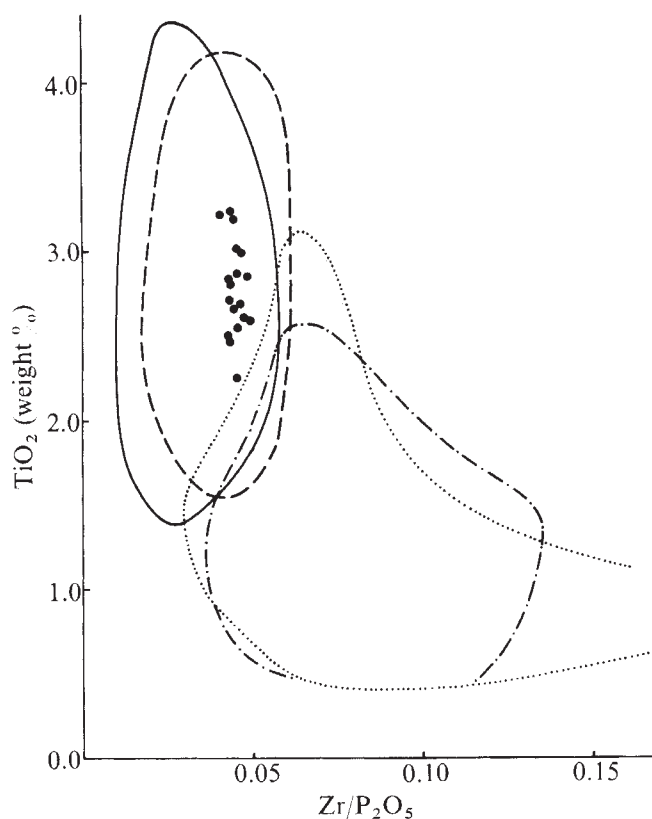


Fig. 2 TiO_2 against $\text{Zr}/\text{P}_2\text{O}_5$ plot for North Sea volcanic rocks. Field boundaries after Floyd and Winchester¹¹: —, continental alkali basalts; ---, oceanic alkali basalts; ···, continental tholeiitic basalts; - · - ·, oceanic tholeiitic basalts.

an active spreading axis this northward extension could have terminated²¹ at the edge of the continental crust against the Bay of Biscay-Labrador Sea transform fault. It is generally believed that no spreading axis existed north of this transform fault at least until the late Cretaceous, when it emerged west of the UK continental shelf and offset along the transform fault. If the embryonic spreading axis (or the underlying feature which gave rise to it) to the west of Spain in Jurassic times were projected northwards below the continental crust, it would pass underneath the North Sea, and it could be significant that the North Sea volcanic rocks occur at the intersection of this extension with the Fair Isle-Elbe wrench fault⁴. Since any offset along the Bay of Biscay-Labrador Sea transform fault would be insignificant until new oceanic crust began to be generated by the mature spreading axis, such a linear projection does not seem unreasonable. What is proposed, therefore, is that from its inception during, or possibly before, the Middle Jurassic, the spreading axis north of the Azores (or its underlying parent feature) extended northwards beneath the continental crust at least as far as the North Sea,

and possibly as far as the hinge point for the opening of the central Atlantic which was situated in south Norway^{20,21}. Activity along this northward extension was insufficient to produce a splitting of the crust but enough to contribute to an approximately east-west tensional regime with consequent thinning of the crust and reactivation of rifting in the North Sea area. Though little or no new crust was generated along this extension (the spreading axis was only just becoming established west of Spain), the uprise of heat in the asthenosphere below the North Sea could have generated the volcanic rocks by the partial fusion of the mantle, with the ensuing magma reaching the surface through the thinned crust along lines of structural weakness—the Fair Isle-Elbe line in this particular case. As the Atlantic continued to spread, the continental crust underlying the British Isles was dragged eastwards by the Eurasian plate as a trailing plate, and the extension of the spreading axis below the continental crust underwent a relative westward migration, becoming more and more offset along the Bay of Biscay-Labrador Sea transform fault. During the Cretaceous this westward migration reached the eastern edge of the Rockall Trough. By the time of Anomaly 24, the migration had moved the spreading axis to the west of the Rockall Bank and the well documented opening of the North Atlantic between Greenland and Europe began.

Our model, though admittedly speculative, involves a deep-seated feature of the type generally associated with the genesis of oceanic volcanic rocks, and that, together with the thinned overlying continental crust, could conceivably account for the nature of the North Sea volcanic rocks. The model is also consistent with a tensional regime in the Jurassic North Sea Basin and its subsequent transition to a compressional regime during the late Cretaceous⁴.

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letters to nature

Direct observation of pulsar microstructure

HANKINS' observations¹ of pulsars exhibiting structure on time scales as short as a few μs have set stringent requirements on the theories which attempt to explain the pulsar emission mechanism^{2,3}. These microstructure observa-

tions^{1,4,5} were made at low radio frequencies where pulsar intensities are high. To overcome the effects of interstellar dispersion, which effectively 'smear out' the microstructure, Hankins developed a complex computation method. Since the effects of dispersion are less pronounced at higher frequencies, microstructure should be directly observable

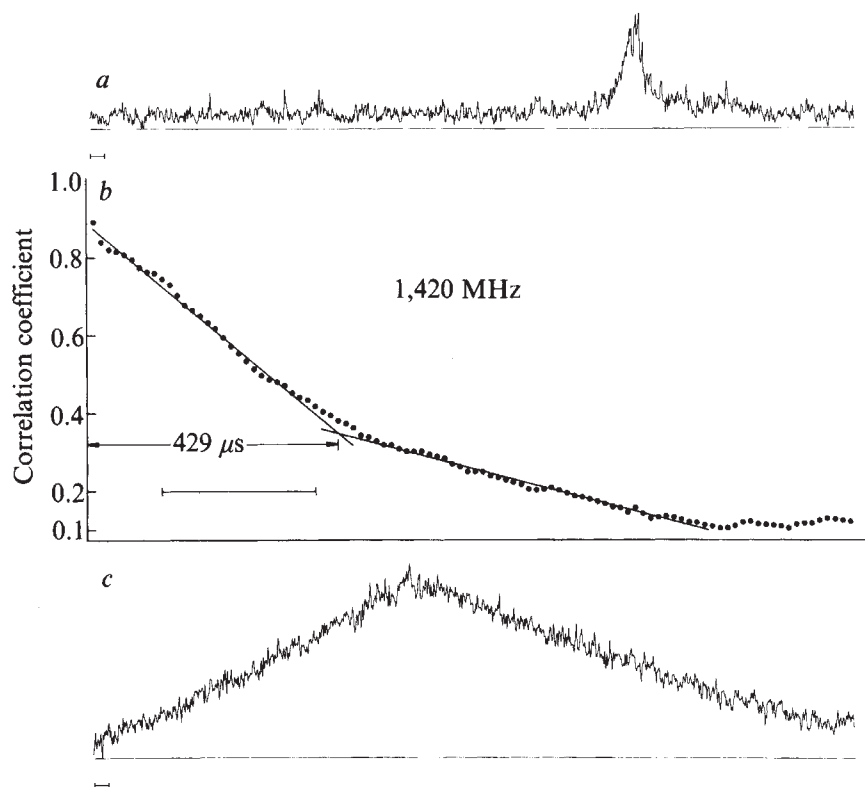


Fig. 1 *a*, A single pulse from PSR1133+16 with a time resolution of $14 \mu\text{s}$; *b*, its autocorrelation function; *c*, the associated integrated pulse shape obtained by adding 2,099 pulses. In all of our figures, the time scale is indicated by the $260\text{-}\mu\text{s}$ bars underneath.

using an appropriate bandwidth. This letter reports high-frequency observations of PSR1133+16 where the dispersion broadening and the time resolution were short enough to reveal microstructure on a time scale of $14 \mu\text{s}$.

We used the 100-m radiotelescope of the Max-Planck-Institut für Radioastronomie at Effelsberg⁶ at an observing frequency of 1,420 MHz, with beamwidth $9'$ and antenna performance 1.5 K Jy^{-1} . The signal was detected with a linearly polarised feed at the prime focus. The first stage of the receiving system was a cooled parametric amplifier giving an overall system noise temperature between 50 K and 65 K, depending on elevation. For pulsar microstructure studies, various filters at the intermediate frequency were available to allow the correct bandwidth to be chosen for the optimum resolution for each pulsar. After detection the signal was digitised by a voltage-frequency converter (with a maximum output frequency of 15 MHz), in conjunction with the pulsar data logger (PDL). This device enables the storage of 1,024 numbers per pulse period, and is controlled by two computers. The first, a Ferranti Argus 500, provides Doppler-corrected pulsar period and timing signals.

The second, a Honeywell H316, takes the data or buffer storage and transfers them through the Argus 500 computer on to magnetic tape.

A number of pulsars were recorded with various resolutions on November 17 and 25, 1975. Observations of the pulsar PSR1133+16 were made with a resolution of $13 \mu\text{s}$. The bandwidth used was 1 MHz, which in view of the pulsar DM of 4.85 pc cm^{-3} , resulted in a dispersion broadening of $14 \mu\text{s}$. The data from each pulse were recorded using 1,000 resolution intervals, each $13 \mu\text{s}$ long. Most of the observations were restricted to the first component of the double-pulse profile.

Our results for both components of PSR1133+16 confirm the general features of the microstructure as described by Hankins^{1,4}. Autocorrelation functions (ACFs) of the strong pulses often show the 'linear' feature associated with microstructure, extending from near zero lag to a few hundred μs . This is followed by a much broader feature associated with the subpulse width. Many pulses lack the microstructure feature in their ACFs which seems to imply either that microstructure is not always an important part of the

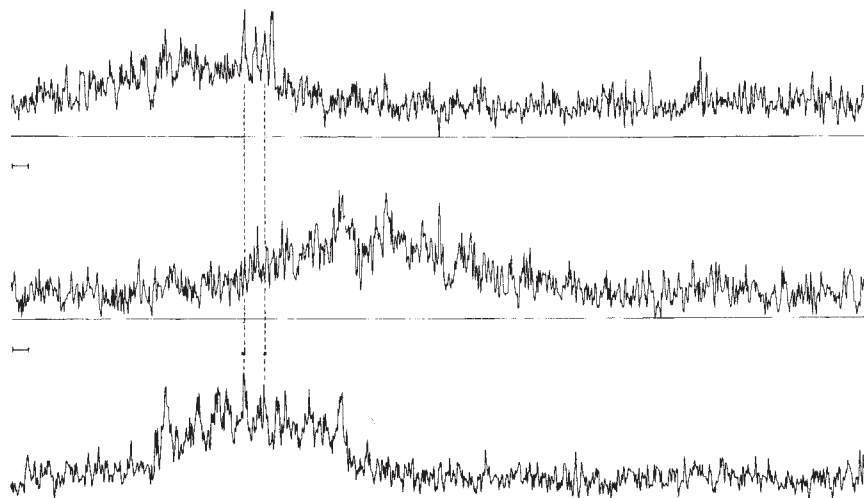


Fig. 2 Three consecutive pulses from PSR1133+16 showing a quasi-periodic behaviour within the subpulses.

emission, or that, if it is present, it is sometimes totally unresolved at 14- μ s resolution. Spikes which are comparable in width with our time resolution are rare, however, and seem to be relatively weaker than those reported at 111.5 MHz by Hankins and Cordes⁵. The flux intensity of the strong pulses in our observations reaches a maximum of 200 Jy.

A pulse (taken from the first component of the integrated profile), which shows microstructure, its ACF and the integrated pulse shape obtained from it and 2,099 surrounding pulses are shown in Fig. 1a, b and c respectively.

An interesting feature which appears in some pulses is a quasi-periodic behaviour within the subpulse. Three consecutive pulses showing this behaviour in their ACFs are shown in Fig. 2. The apparent periodicity is 150 μ s. Hankins remarked that similar behaviour sometimes appears in PSR0950+08, but to our knowledge it has never before been reported for PSR1133+16. Furthermore, the fact that some peaks occur at the same phase in the two strongest pulses, which have subpulse peaks at different phases, makes it uniquely interesting. Of course, this quasi-periodicity appears in the ACFs as secondary maxima at the lags corresponding to the 'period' and its multiples.

Studies of these observations of PSR1133+16, as well as 20- μ s resolution observations already obtained for PSR0950+08 and observations of several other pulsars at resolutions <200 μ s are still in progress and should yield further information on what is the basic pulsar emission process.

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A possible large-scale meridional structure in the helioequatorial solar wind

OVER the past few years observational data for a large-scale meridional structure of the solar wind have been accumulated¹⁻³. Space-probe measurements have revealed heliolatitudinal effects at 1 AU, but little is known about the wind flow pattern at small radial distances, just outside the corona. Here we report the existence of a solar equatorial belt, from which few protons are emitted, and we discuss its causes.

We studied the semi-annual variations in solar cosmic ray propagation with the change of heliolatitude of the Earth. The probability of the arrival of solar cosmic rays at the Earth may be used to measure the conditions for the propagation of solar particles in space, between the solar flare and the Earth. This probability is proportional to the ratio of the proton flux ($E_p > 10$ MeV) to the energy of a solar radio burst (2,800–9,400-MHz range)⁴. A list of proton flares⁴ was used to calculate this ratio averaged over 30 d. In Fig. 1a the probability K , determined in this way, as a function of the Earth's heliolatitude for 1957–63 is presented (182 proton events). One can

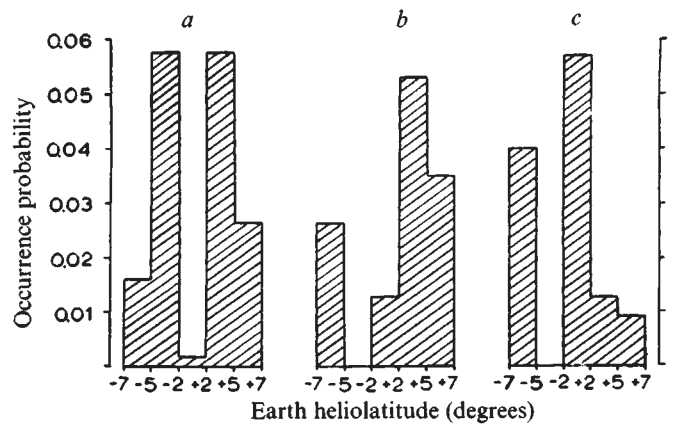
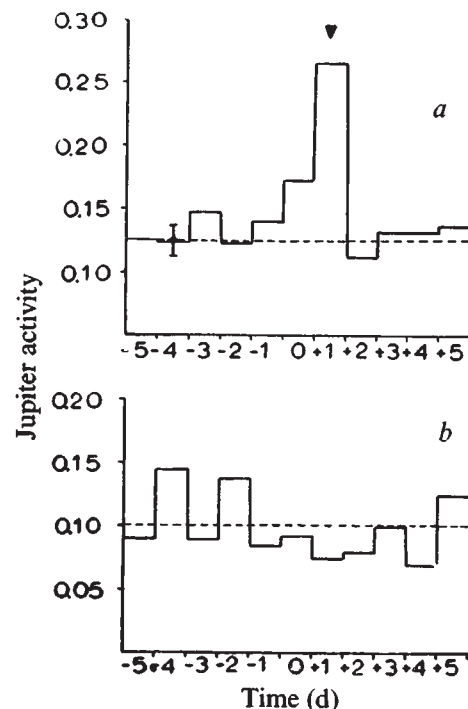


Fig. 1 a, The probability of the occurrence of solar cosmic rays at the Earth, K , as a function of the Earth's heliolatitude (1957–63). b and c, The displacement of the avoidance zone of solar cosmic rays in 1965–69; b, Events in the northern hemisphere; c, Events in the southern hemisphere.

see that very few solar protons penetrate a certain 'layer' centred on the solar equatorial plane ($\sim \pm 2^\circ$ of heliolatitude). This avoidance ('quiet') zone in the solar particles propagation was found also in the data for 1938–56 and 1965–69. The same effect is seen when events in the northern and the southern hemispheres are analysed separately. Clearly the avoidance zone does not completely 'isolate' one solar hemisphere from the other, so the particles emitted by flares in the northern hemisphere can penetrate into the southern region and vice versa, but the mean flux of solar protons decreases when particles cross the equatorial plane (for details see ref. 5).

The quiet zone effect was also revealed in the influence of proton flares on Jupiter's decametric radio-burst activity. Some evidence for an association of the period of Jovian activity with the solar type-IV radio bursts was found by Warwick⁶. This correlation was not, however, confirmed later^{7,8}. Data from Boulder⁹⁻¹² were studied again to search for the effect of solar

Fig. 2 Decametric radio-burst activity of Jupiter after the proton flares (using the superposed epoch method). The ordinate gives the mean indices of Jovian activity. a, When the heliolatitude of Jupiter $> 2^\circ$; b, when Jupiter is within the avoidance zone (heliolatitude $< 2^\circ$).



protons on noise from Jupiter for 1960–71. The indices of Jovian activity were calculated for each day of a 4.5-month period near each opposition (for the indices we adopted the ratio of duration of the storm to the full time of the observation, with weight equal to the importance of the storm). The days when solar proton events as well as solar flares with a type-IV radio burst were observed, were taken as the zero days for the superposed epoch method. Two time intervals were considered separately: first, when the heliolatitude of Jupiter is $> 2^\circ$ and second when this latitude is $< 2^\circ$. It is evident from Fig. 2a that Jovian burst activity increases after flares with a delay of 1 d. The effect exceeds 5σ only when Jupiter's heliolatitude is $> 2^\circ$ (see Fig. 2b). Thus the avoidance zone probably exists at distances up to 5 AU (ref. 13).

These results suggest that a 'screen' might exist in the solar equatorial plane, probably a region of increased interplanetary magnetic field. It is possible that the particle density increases in the same layer. Such a regularity in the meridional distribution of the particle density near the Sun ($\gtrsim 5 R_\odot$) has been inferred^{14,15}. Some density excess in the equatorial plane may, however, be expected also at 1 AU. A density excess of 20–30% has perhaps been seen by Hundhausen *et al.*¹⁶. A corresponding decrease of the plasma velocity in the equatorial plane was revealed by the same measurements¹⁶ in agreement with early results¹⁴ and recent cometary data¹⁷. Some heliolatitudinal changes were also observed in the interplanetary magnetic field (for example in refs 18–20).

Our study therefore strongly suggests that the solar equatorial wind has a considerable meridional structure near the Sun: an annular region of quasi-stationary plasma with an increased magnetic-field intensity may be assumed in the equatorial plane. The angular width of this layer is $\sim 3^\circ$ – 4° (as it is seen from the Earth). The radial distance from the Sun is $\gtrsim 5 R_\odot$. This hypothesis is in good agreement with calculations of the dynamics of solar wind expansion^{21–24}, which predict the existence of a meridional component of the solar wind velocity directed towards the equator at a distance of several $R_\odot$ (inside the critical Alfvén point). The magnetic field lines would be squashed into the equatorial plane (see also ref. 25).

The displacement of the observed structure to the south ($\sim 3^\circ$) for 1965–67 was revealed by the finding of a very large N–S asymmetry in the distribution of solar activity²⁶. A possible displacement of the lines of wind flow in such an asymmetry was suggested in ref. 27. The location of the avoidance zone is shown in Fig. 1b and c (for details see ref. 28). The result obtained agrees with the findings of other authors^{15,29,30} and with the calculations^{33,34} based on the space-probe measurements^{31,32}.

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Solar constant during a glaciation

The causes of ice ages are of great topical and scientific interest. Here we review some recent work on the subject, point out some severe objections to postulated mechanisms, and make our own proposal that the driving force is some periodic behaviour in the Sun's interior.

Kukla¹ has revived the Milankovitch mechanism for glaciation, and values of the solar constant at the autumnal equinox suggest that the climatic optimum of 6,000 yr ago may be accounted for by a run of warm autumns. If the Milankovitch curve^{2,3} is compared with the climatic factors obtainable from magnetically dated deep-sea cores, however, it is clear that this mechanism cannot cause the major glacial cycles (see Fig. 1 and refs 4 and 5). The major variations of the aridity of the land north of Dakar, the trade wind vigour and the ^{18}O glacial stages are in good correlation. There are ~ 8 such cycles down to the 0.7-Myr magnetic date, but, in the Milankovitch curve there are 15 occasions in this period when the summertime radiation at 65°N is significantly below average. At best, the Milankovitch mechanism may be responsible for the minor ripples superimposed on the major trends.

An attractive theory of glacial cycles has recently been proffered by McCrea⁶. He supposes that the Sun passes through a series of very dense dust clouds which border the concave edge of the galactic spiral arms. In a cloud, the Sun accretes matter and its luminosity could increase to high values. McCrea relates these 50,000-yr periods to the glacial stages, supporting Simpson's hypothesis that the warmer Sun provides sufficient precipitation to support a glaciation⁷. This must, however, be viewed with suspicion because it is now established that temperatures fell during a glaciation, in the tropics and at the poles. Furthermore, geological evidence⁸ shows that centres of glaciation were the Scandinavian highlands, the Labrador plateau and other moist mountainous regions. Today these regions receive copious precipitation, yet no continental ice caps grow; probably because they are not cold enough. The McCrea mechanism is essentially one of random occurrence; yet, particularly from core evidence, the climate seems cyclical—crudely sinusoidal, with perhaps systematically increasing amplitude and period for the later cycles^{4,5}.

I suggest that some solar periodicity is the prime cause of the major climatic cycles, and try here to estimate (using Houghton's radiation curves⁹) the Earth's radiation and heat budget that would be in balance with a glacial climate. This leads to a new value of the solar constant which I assume relates to the luminosity of the Sun.

It is difficult to calculate glacial temperatures. Wilson and Hendy¹⁰ have reviewed the ^{18}O data and believe that if the equatorial regions fell by 5°C the polar regions would fall by $\sim 14^\circ\text{C}$. The extensive work on foraminifera in Atlantic cores by the CLIMAP group¹¹ shows that 18,000 yr ago the surface waters, from Newfoundland to the Bay of Biscay were colder by 10°C and that most of the tropical waters were colder by 4°C . Botanical studies in Europe¹² have established the July 10.5°C isotherm during the Würm glaciation as running in the general direction

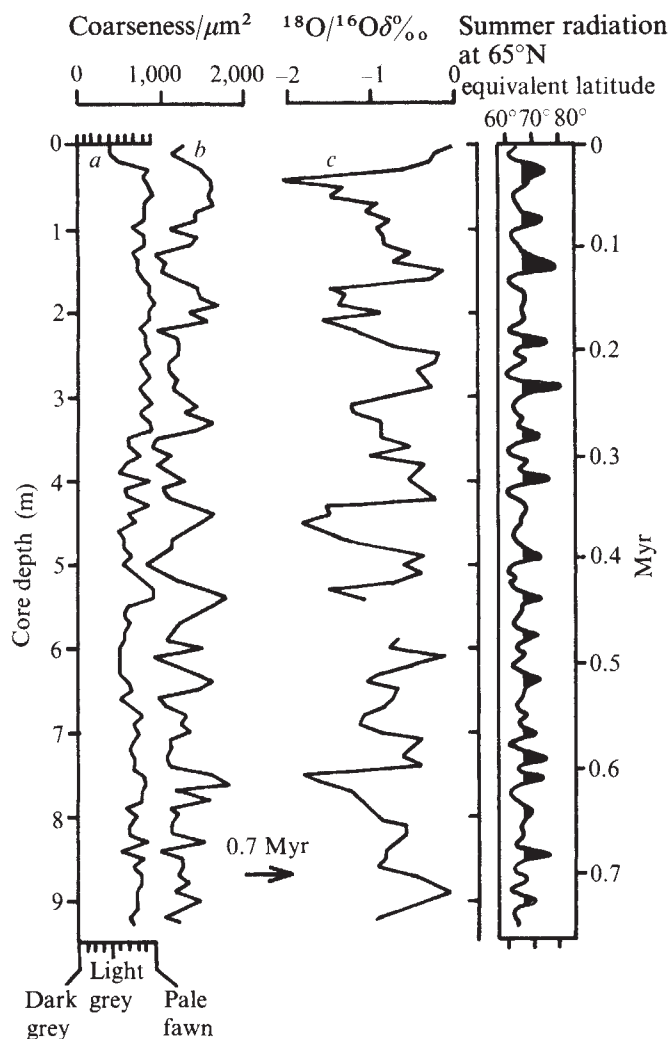


Fig. 1 The comparison between the climate factors down core V23-100 (from 21°18'N, 22°41'W, off the Sahara coast, just north of the Cape Verde Is.) and the Milankovitch curve. *a*, The colour is a measure of vegetation on the Spanish Sahara. The general lack of greyness means that humus (or burnt vegetation) was absent in the soil, that is, desert conditions prevailed throughout the whole time span. *b*, The coarseness is a measure of the size distribution of quartz particles. A high value means strong trade winds, capable of transporting large particles. *c*, The ^{18}O deviations from present-day values measure the ice volume and establish the glacial stages. Note that strong winds and a slight increase in aridity occur during glacials.

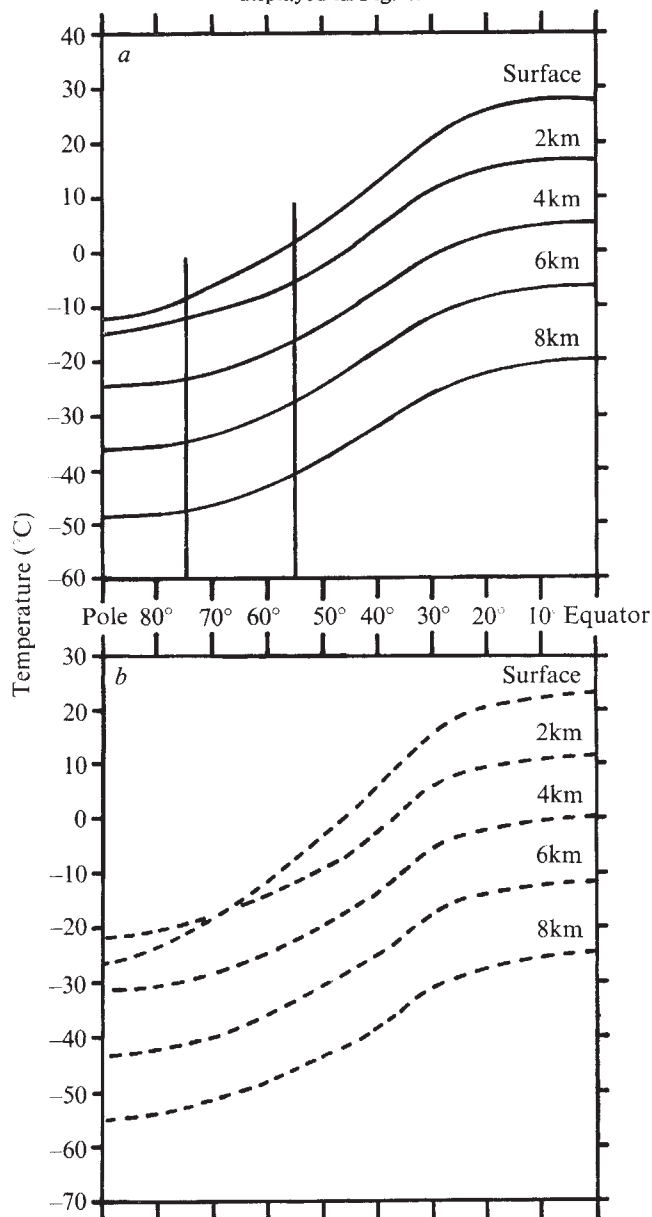
Bordeaux-Berlin-Warsaw-Moscow. Today this line is the 18.5 °C July isotherm. By determining the degree of racemisation of aspartic acid in fossil bones, Schroeder and Bada¹³ have estimated falls of 4 °C for the Mediterranean coast and 5–6 °C for East Africa. Judging drops in temperature from the lowering of the snow line is uncertain, as it depends markedly on precipitation; but assuming no change for Mount Kilimanjaro then an ~ 5 °C fall⁸ would be reasonable for equatorial mid-tropospheric air. In estimating the long-wave radiation back to space, I generalise these data and assume surface level falls of 14 °C polewards of 80°N, 11 °C between 80°N and 60°N, 8 °C between 60°N and 40°N, and 5 °C from 40°N to the Equator.

In Fig. 2*a*, annual temperature profiles are shown at regular levels in the troposphere. The present-day ice cap is taken at a mean latitude of 75°N and the glacial ice cap at a mean latitude of 55°N (see ref. 7). This itself suggests a surface fall of 11 °C ~70°N, but a fall of only 6 °C for the mid-tropospheric air. In Fig. 3*a*, Houghton's annual absorbed solar radiations and re-emitted infrared radiations are reproduced (continuous lines), and the total heat flux

across the circles of latitude are shown in Fig. 3*b*. Plotting the infrared radiation against the surface level temperature profile (Fig. 4), a linear relationship is obtained from the pole to ~ 20°N; however, against the upper air temperatures (say at 4 km) the empirical relationship is more complex. In the doldrum belt, towering cumulus cloud probably accounts for the 10°N and equatorial points plotting below the straight line. To estimate the infrared radiation to space during a glaciation, I assume that the present-day latitude points can be moved, parallel to the empirical line, by the appropriate temperature fall assumed for that latitude. This will tend to include the effects of the temperature-dependent changes in the concentration of water vapour¹⁴. The glacial infrared radiations are transferred to Fig. 3*a* as the dashed line.

Before the solar radiation curve can be proportionally reduced to secure a balance, it must be corrected for obvious changes in the planetary albedo. The present-day albedo (the inset in Fig. 3*a*) is redrawn along the dashed curve 1. This assumes that the glacial albedo at 55°N is the

Fig. 2 *a*, Present-day temperature profiles, constructed from mean meridional cross sections (Arctic Forecast Guide, US Navy Weather Research Facility, April 1962). The vertical lines give the position of the ice caps. *b*, Glacial temperature profiles. The surface level is based on currently available observational data; whereas the upper levels depend on the reliability of the method displayed in Fig. 4.



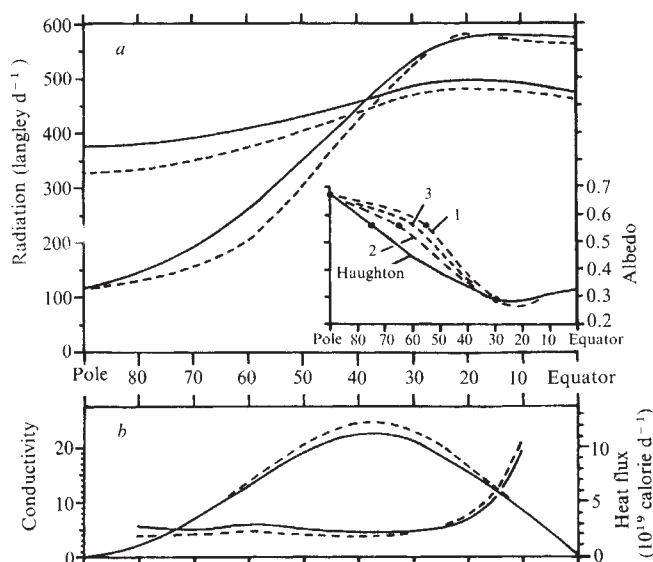
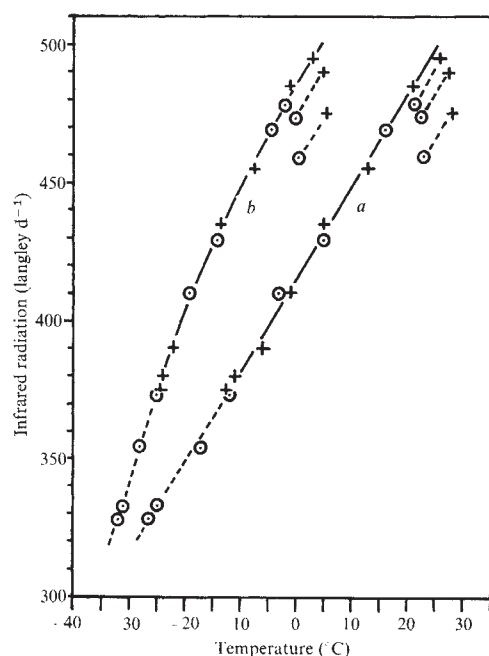


Fig. 3 *a*, The Houghton radiation curves, and the estimated radiation curves that are in balance during a glaciation for a 2.5% reduction in the solar constant. The inset shows the Houghton planetary albedo and the three glacial albedos discussed in the text. *b*, The Houghton heat flux and the estimated glacial heat flux across the circles of latitude. The latitudinal conductivity is also shown. Continuous lines refer to present-day values, dashed lines to glacial values. (1 langley = 1 g calorie cm⁻²). The latitudinal thermal conductivity = (heat flux per cm length of the latitude circle)/(thermal gradient, as °C per degree latitude).

same as that now at 75°N. I think this is an overestimation, and I believe it could not be made any greater. Evidence from the deep-sea core (Fig. 1) suggests that the Spanish Sahara has always remained desert and, if anything, was more arid (had less cloud cover) during glacials. Extending these results to the globe, it therefore seems reasonable to assume that at 30°N the albedo is unchanged, but perhaps at 20°N it could be reduced slightly. The pole and the equatorial regions are assumed to have a constant albedo.

Fig. 4 The relationship between the surface-level temperature profile (*a*), the 4-km profile (*b*), and the infrared radiation emitted back to space at the 10° latitude intervals from the Pole to the Equator. (+, Present-day values, ⊙, the estimated glacial values—see text.)



With this first albedo assumption, the solar radiation curve is replotted (not shown), and then it has to be reduced by 1% to give a balance. The heat flux, across the latitudes, plots (not shown) well above the present-day value, except polewards of 65°N. I feel that the glacial heat flux should be greater than the present flux, but at all latitudes; clearly this first albedo is too high. The second albedo curve is deliberately drawn too low, making 65°N equivalent to 75°N. Balance is obtained with a 4% reduction, but the heat flux plots everywhere just below the present values. Some intermediate albedo is more realistic and I choose curve 3, which gives a balance for a 2.5% reduction; and the heat flux (the dashed curve in Fig. 3*b*) is everywhere greater than the present day. This glacial absorbed solar radiation curve is shown in Fig. 3*a*. If the albedo difference at 20°N is ignored, then there would be a 2% reduction. Assuming that present-day conditions are midway between a full interglacial and a full glacial, then I venture to suggest that the luminosity of the Sun has fluctuated with a 5% amplitude range and period of 10⁵ yr.

It may be possible to reconstruct the temperature profiles for a glacial climate. Taking the 4-km profile as an example, the ten latitude points are moved along the empirical curve until they are at the appropriate glacial infrared radiation value, determined from the ground-level line. This gives a drop of 7 °C for the pole and for 80°N, 6 °C for 70°N, but a uniform fall of ~ 5.5 °C for the other points. In our present-day troposphere the average lapse rate of 5 or 6 °C km⁻¹ is constant everywhere above 2 km, and does not depend on season. If the same lapse rate persists in a glacial climate, profiles at various heights may be constructed (Fig. 2*b*). A strong inversion between the surface and a height of 2 km appears polewards of 70°N as an annual feature.

It is interesting to compute, from the profiles, latitudinal thermal 'conductivity'. Physically, this would be a measure of the meridional component in the wind and ocean current structure. Using the surface-level profiles, the present-day and glacial conductivities are given in Fig. 3*b*. The high value in the tropics probably relates to the Hadley cell; but in the westerly wind belt, the meridionality of the transporting system is much reduced, and it seems that the glacial westerlies and ocean currents were noticeably more zonal than those of the present day.

It is interesting to note that the CLIMAP results do indicate a displacement of the Gulf Stream south of its present course, as if strong zonal winds directed it, during a glacial, more towards the Azores. Of course, the assumed increase in thermal gradient over the middle latitudes would encourage an eastward vector in the westerlies; and, treating the atmosphere as a heat engine, the whole circulation would have to be more vigorous. Evidence for this increase in vigour is already apparent in the deep-sea core work, some of which strongly suggests enhanced monsoonal activity^{4,5}. Perhaps the Azores anticyclone intensifies, moves south-eastwards, and effectively blocks the Spanish Sahara from encroaching westerlies. A simultaneous intensification of the St Helena high would give an added 'push' to the monsoon.

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Far infrared emission spectrum of the stratosphere from balloon altitudes

WE present here observations of the far infrared emission spectrum of the stratosphere taken with a balloon-borne interferometer from an altitude of 22 km above Churchill, Manitoba, Canada (latitude 58.7°N, longitude 94°W) in July, 1974. The instrument was part of a composite Atmospheric Environment Service of Canada gondola whose overall aim was the measurement, by several complementary methods, of the concentrations of minor constituents of the atmosphere and the monitoring of their changes with altitude and time during the flight. Many of the minor constituents of particular interest in the current debate on stratospheric pollution (O_3 , H_2O , NO_2 , N_2O , NO , HNO_3 , HCl , SO_2) exhibit pure rotation spectra in the far infrared and are expected to contribute to the stratospheric emission spectrum. O_2 , a well known and well behaved constituent of the stratosphere emits a series of weak magnetic dipole lines in this wavelength region, with intensities matching those of intrinsically stronger ones from less abundant species, and which can be used for spectral normalisation (see ref. 3).

Our preliminary data have been analysed by a method which uses synthetic spectra to allow for the weak O_3 lines and to effect a correct normalisation of the observations to the O_2 lines. The synthetic spectra were produced by a program described by McClatchey *et al.*¹, modified to use the instrumental convolution functions $\text{sinc } x$ or $\text{sinc}^2 x$ more applicable to Fourier transform spectroscopy (see ref. 2), with magnetic dipole transitions of O_2 added.

We used a simple rapid-scanning Michelson interferometer with no phase modulation, which produced interferograms with fringe frequencies for the highest wavenumbers of interest ($\sim 150 \text{ cm}^{-1}$) matching the maximum frequency response of the Ge(Ga) bolometer detector system² at Infra-Red Laboratories, Tucson, Arizona ($\sim 20 \text{ Hz}$).

The design of the system provided reproducibility between spectra in the wavenumber range $30\text{--}130 \text{ cm}^{-1}$ to a resolution of 0.2 cm^{-1} at least equal to that of data taken from aircraft altitudes at equivalent resolution by phase modulation techniques^{4–6}. Some of the more relevant details of the instrument and of the flight are discussed in the caption to Fig. 1, which shows a composite of two single spectra, displaced vertically to facilitate comparison.

Spectra were produced sufficiently rapidly that adequate height resolution during ascent (about 200 m per spectrum) and time resolution during sunrise and sunset (about 70 s per spectrum) were provided. Spectra could then be averaged if necessary after the flight to produce better signal-to-noise ratios. These spectra agree closely with those from aircraft altitudes^{4–6}, but with important differences. Strong H_2O lines which dominate most of this spectral region are considerably weaker at balloon altitudes. The O_2 lines, though still easily discernible up to 80 cm^{-1} , are somewhat weaker, particularly when compared with the comparatively strong Q branch emissions of O_3 . This is as expected from the distributions of those constituents in the atmosphere. Weak O_3 lines pervade the whole region up to about 70 cm^{-1} and produce much of the small scale structure, making weak emissions from other constituents difficult to distinguish at this resolution. Strong H_2O lines still exhibit considerable emission in the wings of the lines, particularly those at 55, 56 and 88 cm^{-1} . No lines of H_2O other than

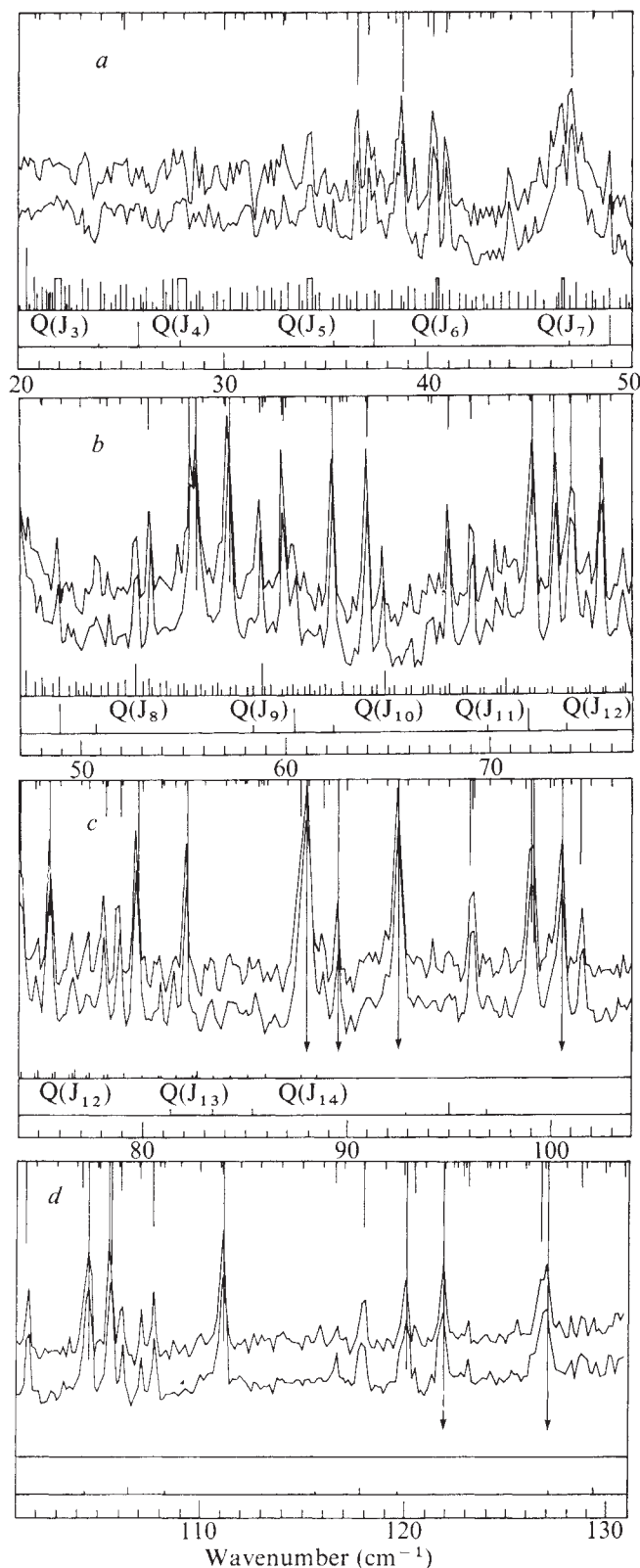


Fig. 1 Two emission spectra of the stratosphere between 20 and 130 cm^{-1} (resolution 0.2 cm^{-1}), taken at a zenith angle of 75° , from about 22 km in the anti-Sun azimuthal direction at 01.05 UT, July 23, 1974 (20.05 LT). a, $20\text{--}50 \text{ cm}^{-1}$; b, $50\text{--}75 \text{ cm}^{-1}$; c, $75\text{--}100 \text{ cm}^{-1}$; d, $100\text{--}130 \text{ cm}^{-1}$. H_2O line positions and relative strengths are shown above the spectra; O_3 lines and Q branches are delineated immediately below the spectra. Magnetic dipole lines of O_2 are depicted below the O_3 line positions. The Ge(Ga) bolometer detector was fed by an $f/1.5$ TPX lens of 25 mm diameter, and was filtered by a mesh filter (at 2 K) a PTFE blocking filter (2 K), 0.4 mm black polyethylene ($\sim 100 \text{ K}$) and a TPX window at 253 K. The interferometer beamsplitter was a $12\text{-}\mu\text{m}$ thick Mylar (du Pont).

from the most abundant isotopic species are immediately obvious, nor is there evidence of emission from the dimer of H_2O , even under very careful analysis using synthetic spectra in regions of expected emissions. This finding is contrary to previous reports of aircraft measurements^{7,8}.

There is little evidence of emissions from other minor constituents in the preliminary analysis of these spectra. A peak is evident on an expanded plot at 37.8 cm^{-1} in the predicted position of a pseudo-Q branch of NO_2 (ref. 9). These emissions have been identified by Harries *et al.*¹⁰ on spectra taken at aircraft altitudes, and a value for the integrated column density has been determined for that altitude. No peak of comparable magnitude to that observed in our spectra is predicted by the synthetic spectrum which includes O_2 , H_2O and O_3 emissions. The line strength for this NO_2 emission is poorly known, however, and mixing ratios derived from this line are considerably above those obtained from other instruments on this flight. More definitive identification of the presence of emissions from this constituent at these altitudes must await spectra of higher resolution.

Of the expected HCl emission line positions only two are comparatively free of emission from other constituents, those at 41.6 cm^{-1} and 83.3 cm^{-1} . Expanded portions of the spectra at these wavenumbers indicate excess emission over that expected from weak O_3 lines as indicated by the synthetic spectrum, but it is not possible to say definitively whether emissions originate with HCl molecules. An upper limit to the mixing ratio of HCl on the basis of these data would be several parts per 10^9 , considerably higher than the upper limit of 0.18 parts per 10^9 , placed on this constituent in the lower stratosphere by Farmer¹¹ from near infrared observations.

Considerable structure is seen in the spectra between strong lines beyond about 80 cm^{-1} where weak O_3 lines should be negligible, and other molecular species are being examined as sources of these small but significant peaks.

The integrated column densities of H_2O and O_3 were obtained, and from several combinations of lines near to Q branches of O_3 the column densities of H_2O and O_3 above 22 km were determined as $1.1 (\pm 0.6) \times 10^{18}$ and $5(\pm 2) \times 10^{18}$ molecules per cm^2 , respectively. Further analysis of this kind, with careful fitting of data to synthetic spectra, particularly those calculated using multi-layer atmospheric models, should yield more precise values of these constituents in the stratosphere. It is apparent, however, that values of line strengths for the magnetic dipole transitions of O_2 are needed to greater precision than are at present available (see ref. 12), particularly at the higher wavenumbers.

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Lamb waves originating in non-geostrophic perturbations

A LAMB wave is a purely compressional wave mode in an isothermal atmosphere at rest above a horizontal rigid surface¹. It travels only horizontally, with a phase velocity equal to the speed of sound and its energy density decreases exponentially with height. Bretherton² and Garret³ have concluded that such a mode may exist in the temperature- and wind-stratified atmosphere of the Earth, with its energy mainly located at heights below 30 km. Lamb waves belong to the family of acoustic-gravity waves which, according to Rossby⁴ and Obukhov⁵, may be excited by non-geostrophic perturbations in a rotating stratified fluid until the fluid has returned to a quasi-geostrophic steady state. Here we report the observation of Lamb-wave generation in the troposphere during a geostrophic adjustment process.

On several days before and after the solar eclipse of June 30, 1973, simultaneous atmospheric and ionospheric observations were made at Tsumeb (19.2°S , 17.7°E) in southern Africa. On July 1, a microbarograph recorded unusually large ground-level pressure fluctuations, which, of course, were not caused by the eclipse. They have periods of $\sim 1\text{ h}$, and take place from morning until evening. Figure 1 shows a digitised form of the pressure record, but it is

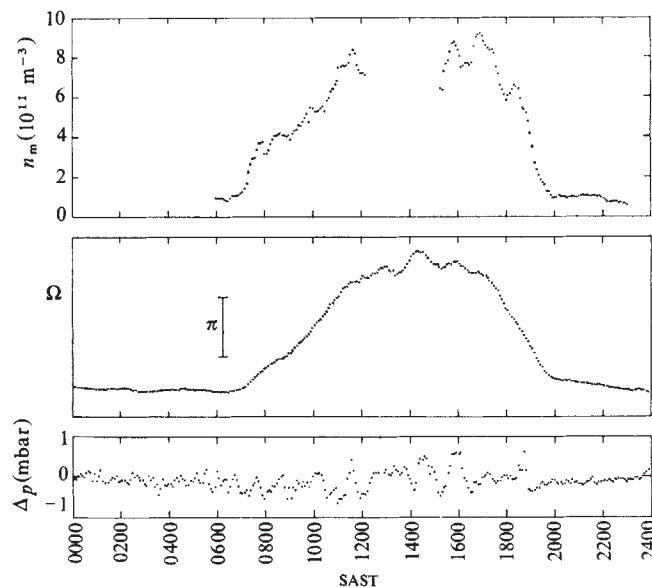


Fig. 1 Ground-level pressure variation Δp , Faraday rotation angle Ω of the 137-MHz radio signal of the geostationary satellite Intelsat IIF3, and maximum electron density n_m against South African Standard Time at Tsumeb on July 1, 1973.

influenced by the filter characteristic of the microbarograph. Similar fluctuations superposed on the diurnal variations occurred in the Faraday rotation angle of the radio signal of a geostationary satellite and in the maximum electron density of the F2 layer (Fig. 1). The Faraday rotation is a measure of the ionospheric electron content which usually reacts very insensitively to perturbations in the electron gas. The maximum electron density is at a height of $\sim 230\text{ km}$ and was deduced from vertical incidence ionograms which were not, however, available between 1210 and 1515 SAST. Around 1200 and from 1515–1800 SAST, it is particularly obvious that the electron density fluctuations show a strong correlation with the pressure fluctuations. A power spectrum analysis of all records shows two significant oscillations at periods of 90 and 60 min respectively, and each oscillation

has been isolated by a numerical bandpass filter. The two filters are characterised by centre periods of 90 and 60 min, and half-power widths of 38 and 18 min, respectively. Figure 2, for instance, shows the isolated 90-min oscillations confirming the strong correlation between the tropospheric and ionospheric events.

Additional Faraday rotation records were made at Outjo (20.1°S, 16.2°E) and Okahandja (22.0°S, 16.9°E), at distances ~250 km from Tsumeb. The filtered Faraday rotation data at all three stations show different phase angles, indicating that the oscillations are caused by propagating waves. For both periods, we find northward horizontal phase trace velocities of 220 m s^{-1} . The observed periods and velocities are typical of acoustic-gravity waves which propagate in the neutral gas causing perturbations of the ionised gas by ion-neutral collisions and by enhanced production and loss of ionisation^{6,7}.

The waves have been traced back towards the south by means of further Faraday rotation records and ionograms taken at several South African stations—additional microbarograms were not available. The 90-min wave could not be detected at Hermanus (34.4°S, 19.2°E), the 60-min wave not at Grahamstown (33.3°S, 26.5°E). Thus we may conclude that the waves were excited at low heights near these stations and could not reach the ionosphere above because their energy propagated on inclined ray trajectories⁸. Both stations are near the southern coast of Africa where, at the time in question, considerable non-geostrophic winds occurred (Fig. 3). Additional weather maps indicate that these winds were strongest during the daylight hours of July 1. The close spatial and temporal coincidence of the observed waves and ageostrophic winds thus confirms the theory that acoustic-gravity waves may originate in non-geostrophic perturbations.

It is reasonable to expect that the atmospheric response to a tropospheric source is dominated by Lamb waves because their energy is mainly located at low heights^{9,10}. To test this hypothesis we shall compare our observations with calculated Lamb waves in a realistic atmospheric model. We take the 1972 Mean COSPAR International Reference Atmosphere which, below a height of 25 km, is

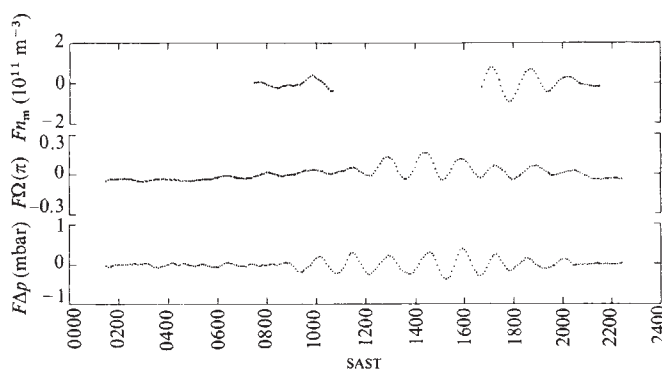


Fig. 2 Filtered records of ground-level pressure, Faraday rotation angle and maximum electron density.

completed by the 1966 US Standard Atmosphere Supplements. Winds are not taken into account since their meridional components, in particular, are unknown. The electron and ion number densities are described by α -Chapman layers with maximum densities of $4 \times 10^{11} \text{ m}^{-3}$ at a height of 230 km. The wave-induced perturbations of the neutral and ionised gas are calculated from the linearised hydrodynamic equations including dissipative effects¹¹. In Table 1, observed and theoretical results are compared where T =wave period, v =horizontal phase trace speed, r_n =relative perturbation of maximum electron density, r_p =relative perturbation of ground-level pressure, ϕ =phase difference between r_n and r_p . A calculation of the perturbation of the Faraday rotation angle is not possible for reasons given by Georges and Hooke¹².

The differences between observed and theoretical values of v most probably arise from the fact that, in deriving v from the Faraday rotation data, a horizontally-stratified ionosphere was assumed. A deviation of only 3° from the horizontal stratification, for instance, would introduce an error of some 100 m s^{-1} in the observed value of v because, at F-region heights, the Lamb waves behave like internal gravity waves with vertical wavelengths roughly five times

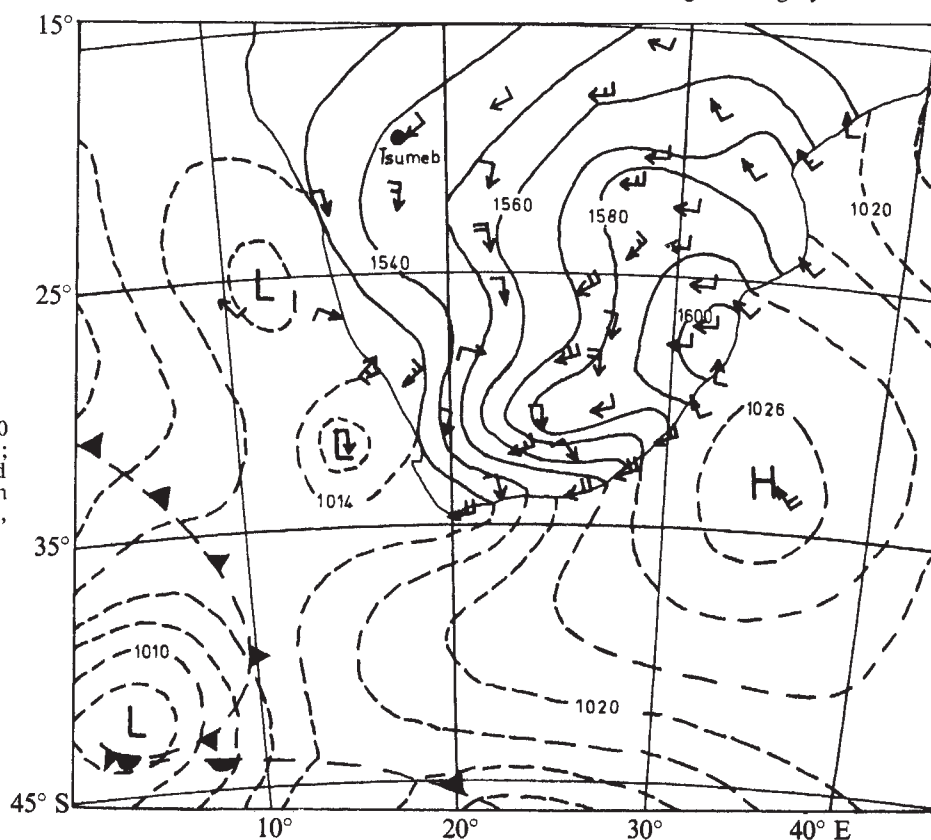


Fig. 3 Weather map on July 1, 1973, 1400 SAST. ---, Isobars at MSL in mbar; —, contours at 850 mbar (gpm); ←, wind direction, each feather = 10 knots. (From Synoptic Weather Map, Weather Bureau, Republic of South Africa.)

Table 1 Comparison between observed and theoretical results

	$T = 90$ min		$T = 60$ min	
	Observed	Theoretical	Observed	Theoretical
v (m s ⁻¹)	220	310	220	310
$ r_n/r_p $ *	550	700	450	770
ϕ * (deg)	65	120	75	100

*Observed pressure perturbations are corrected according to the filter curve of the microbarograph. The values of ϕ have phase ambiguities of $2\pi n$.

smaller than the horizontal wavelengths¹⁰. The differences between the observed and theoretical values of $|r_n/r_p|$ and ϕ , on the other hand, possibly come from simplifications in the theory such as neglect of winds, surface friction and diffusion of the ionised constituents. In particular, the last two processes tend to reduce the differences in $|r_n/r_p|$. In spite of the highly magnified wave amplitudes at thermospheric heights, nonlinear effects play no role.

Other acoustic-gravity wave modes that can propagate in our atmospheric model give no better explanation for the observations than the Lamb mode. In particular, they yield values of $|r_n/r_p|$ which differ from the observed data by more than an order of magnitude. This is because other wave modes are ducted primarily in the upper atmosphere whereas the Lamb waves are guided by the Earth's surface¹³.

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Ni and Co content of chondritic metal

WE have made a detailed study of the metal phases in some chondrites. Axon and Goldstein have reported¹ microprobe measurements of the Ni and Co contents at the interfaces between contiguous α and γ phases in seven metallic particles of high Co content from Apollo 14 and 15 lunar soils. The three particles of highest Co content had α phase in contact with clear homogeneous γ phases (Fig. 1), whereas the particles of lower Co content (Fig. 1) had Widmanstätten spindles of α phase in a matrix of high Ni γ phase in a manner analogous to that observed in certain Ni-rich ataxite irons. Axon and Goldstein have expressed the view that the Co contents of these particles are “higher than the meteorites of similar structure” and that they must have come from a different source.

In two instances, Olivenza (LL5) and Khanpur (LL5), we have encountered spindle-like exsolutions of the high-Co α phase in a matrix of high-Ni-high-Co γ phase. The α - γ interface values for the two meteorites are plotted together with measured bulk compositions in Fig. 1. It can be seen that the meteoritic metal is similar in structure and composition to the lunar soil particles, although the interface composition of the meteoritic γ phase shows less enrichment of Ni. In terms of the binary Fe-Ni equilibrium diagram, the LL5 metal seems to have γ -phase interface

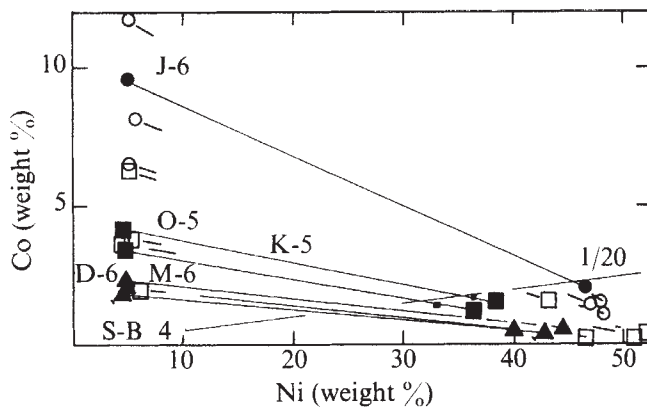


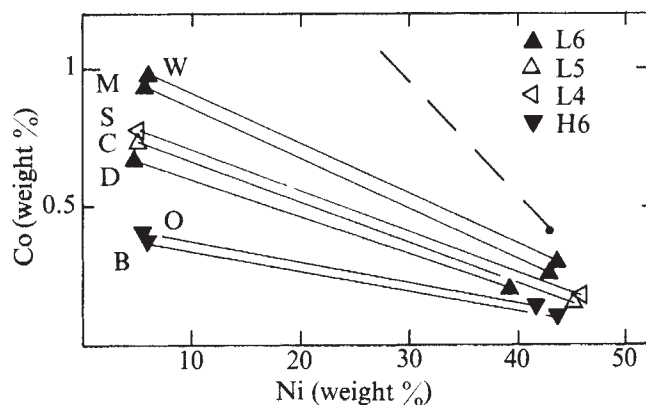
Fig. 1 Interface compositions for α and γ phases in the metal of LL chondrites (solid symbols) compared with high cobalt metal in lunar soils (open symbols). Circles, Clear γ phases; squares, ataxite type structures; triangles = zoned γ phases. J, Jelica; O, Olivenza; K, Khanpur; M, Mangwendi; D, Dhurmsala; S-B, Soko-Banja. Number, petrological grade of LL chondrites.

compositions appropriate to $\sim 410^\circ\text{C}$, in contrast to the value of $\sim 350^\circ\text{C}$ observed for the lunar material.

Figure 1 also includes α - γ interface values for Jelica (LL6). In this meteorite the α phase is extremely rare. When the α and γ phases are contiguous in Jelica, however, the γ phase is clear and compositionally unzoned, and in this case the bulk composition of the metal is almost coincident with the reported γ -phase composition. The bulk metal compositions for Khanpur, Olivenza and Jelica have a Co-Ni ratio of 1:20 (Fig. 1). This represents the cosmic abundance ratio of the two elements. Therefore, the bulk Ni-Co contents of the metal phases in these three LL chondrites are consistent with a Prior's Law relationship in which iron partitions between the metal and silicate phases, whereas the more noble elements Ni and Co concentrate exclusively in the metal. The bulk composition of the metal is first established according to Prior's Law, and the distribution of Ni and Co between the α and γ phases is then controlled by tie-line relationships in the Fe-Ni-Co ternary equilibrium diagram. The lower part of Fig. 1 contains data for Mangwendi (LL6), Dhurmsala (LL6) and Soko-Banja (LL4). In each of these instances the α phase is contiguous with a compositionally zoned γ phase.

None of the samples in Fig. 1 shows visible signs of reheating in the metal phases. They are all observed falls, free from terrestrial corrosion and ablation heating. The silicate portions of these samples have been examined by Sears and Mills² who have given details of identification.

Fig. 2 Interface compositions between α and zoned γ phases in L and H chondrites. The broken line represents the lowest line of Fig. 1. W, Wold Cottage; M, Mauerkirchen; S, Saratov; C, Crumlin; D, Durala; O, Ogi; B, Butsura.



The same criteria were used to select an array of H and L chondrites from Sears' and Mills' samples. The results are shown in Fig. 2. The Ni content of the α phase is essentially the same in both Figs 1 and 2, but there is a distinct Co hiatus of about 1% between the LL α (Fig. 1) and the L α phases (Fig. 2). There is also a marked change in the slope of the Ni-Co tie-lines between Figs 1 and 2, as may be seen from the broken line in Fig. 2, which represents part of the lowest tie-line (Soko-Banja) of Fig. 1.

No metallurgical reheating effects were observed in the samples shown in Fig. 2, but the kamacite (α phase) in both Wold Cottage (L6) and Mauerkirchen (L6) contained minor α - γ shock transformation of the type that Axon and Boustead³ have shown does not alter pre-existing compositions at α - γ interfaces. Within the limits of this investigation, no consistent differences emerged between the petrological grades L4, L5, L6. Any hiatus between the Co contents of α metal in Hand L chondrites is, however, much less pronounced than that between L and LL chondrites. In contrast, the silicate compositions seem to show the reverse effect.

Reheating or shock-heating effects were identified in the metal from three of the samples from ref. 2 that are not plotted in Fig. 2. They are: Holbrook (L6), (α =5.8 Ni, 1.15 Co; γ =29.0 Ni, 0.45 Co); Gambat (L6), (2.86, 0.86; 16.2, 0.26), and Tennasilim (L4) (3.98, 0.75; 25.6, 0.25). Lines joining the α and γ compositions in these reheated samples of metal cross the assembly of Fig. 2 at a steep angle. It is noteworthy that the interface data for the metal in these reheated L chondrites are similar to those reported by Goldstein *et al.*⁴ for two-phase α - γ or α -plessite structures in metal from Apollo 17 soils.

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Non-crystallinity and polymorphism in elemental solids

THIS paper shows a universal correlation of the non-crystalline state with polymorphism in elemental solids. This is demonstrated by a map constructed on a plot of the number of polymorphic forms (NPF) against the group number (GN) for all solid elements in the Periodic Table (Fig. 1). From this result, we propose that both the structure and occurrence of a non-crystalline solid are governed by the structures shown by crystalline states of the element.

The number of polymorphic forms was derived primarily from the data given by Eckerlin and Kandler¹, and by Pearson². High pressure polymorphs were included because localised atomic packings in non-crystalline solids may assume the same bonding configurations as these exhibit. The elements with such high pressure polymorphs are As, Ba, Bi, Dy, Ga, Gd, Ge, Mn, rare earths, P, Pu, S, Sb, Si, Sn, Sr, Tb, Te and Yb. Polymorphs with a similar space group were counted just once, since the bonding natures may be almost identical among similar polymorphic structures, for example, B, Ce, Fe, Ga, Li, Mg, P, Pu, S and Te.

The GNs were arranged to give a progression in degree of bonding anisotropy from isotropic metallic bonding to anisotropic metallic bonding, to covalent bonding, to anisotropic semi-metals and back to isotropic metallic bonding.

Non-crystallinity is favoured in elements with a large number of polymorphic forms and a high degree of bonding anisotropy. Therefore the occurrence of non-crystallinity was described by regions with tie lines bounding the non-crystalline elements formed in similar conditions (see Fig. 1). We thus defined five regions distinguished by the most convenient, or least stringent, conditions for formation. Elements located on the boundary between two regions are best assigned to the lower numbered region.

In region I, non-crystalline solids can be prepared simply by quenching from the liquid state. This region contains the elements S, Se, P, As, and B.

In the upper part of region II, non-crystalline solids can be obtained by vapour, electrolytic or sputter-deposition near room temperature (elements such as C, Si and Ge). For the lower part of the region, Sb, Bi, Te, Fe and Ni require much lower deposition temperatures, ranging from liquid nitrogen to liquid helium temperature. Nickel and Fe were put in region II since they become amorphous in conditions similar to those for Te, Sb, and Bi.

For region III, non-crystalline solids can be formed only by vapour or sputter deposition at very low temperatures (near liquid helium temperature). Region III is defined by metallic elements Mn, Pu, Dy, La, Hf, V, W, Re, and Pd, and semi-metals Pb, Sn, Ge, and Tl. This region includes transition metals with GN from 4b to 8. Bonding characteristics for transition elements do not, however, change as much as the GN indicates, and therefore the occurrence of non-crystalline phases should be more dependent on the NPF than on the GN. Normally, the stability of the non-crystalline solids in region III is enhanced by impurities, thinness, and substrate conditions^{3,4}. We therefore expect that a number of elements on the boundary between regions III and IV, such as Pb, Tl, Pu, Dy, La, Ce, Sm, Yb, U, Np and I to form non-crystalline phases under favourable conditions.

Non-crystalline solids are difficult to form for elements in region IV. Formation requires deposition at very low temperatures in the presence of stabilising factors including contamination and thin film effects, or favourable substrate surfaces. With the scant data available, region IV tentatively includes all group 2a and 3b elements at the left hand of the map. The elements Al, In, Zn, Cd, Hg, Cu, Ag and Au make up region 4 on the right hand of the map. Future work on elements in this region may require extra attention paid to the characterisation of the non-crystalline states as well as the control of experimental conditions, especially for the reported amorphous phases of Al, Be, Zn, Cd, Ag and Au.

There is no evidence for the occurrence of non-crystalline phases in Na, Cs, K and Rb, which make up region V. Because of the isotropic bonding characteristics and small number of polymorphs in group 1a, we believe these elements will not form non-crystalline solids.

This good correlation of polymorphism with the occurrence of elemental non-crystalline solids demonstrates that certain structural aspects of the crystalline state are relevant to the non-crystalline form. We therefore postulate that the structure of a non-crystalline solid is composed of a finite number of bonding states (or polymorphic bonding) with the same bonding characteristics (length, angle, and energy) exhibited by the polymorphs.

It is more reasonable to expect that crystalline state bonds exist in an amorphous solid rather than random packing arrangements. For each thermodynamically stable crystalline structure, there will be a limited number of bonding configurations constituting the lowest free energy state. The formation of non-crystalline phases, however,

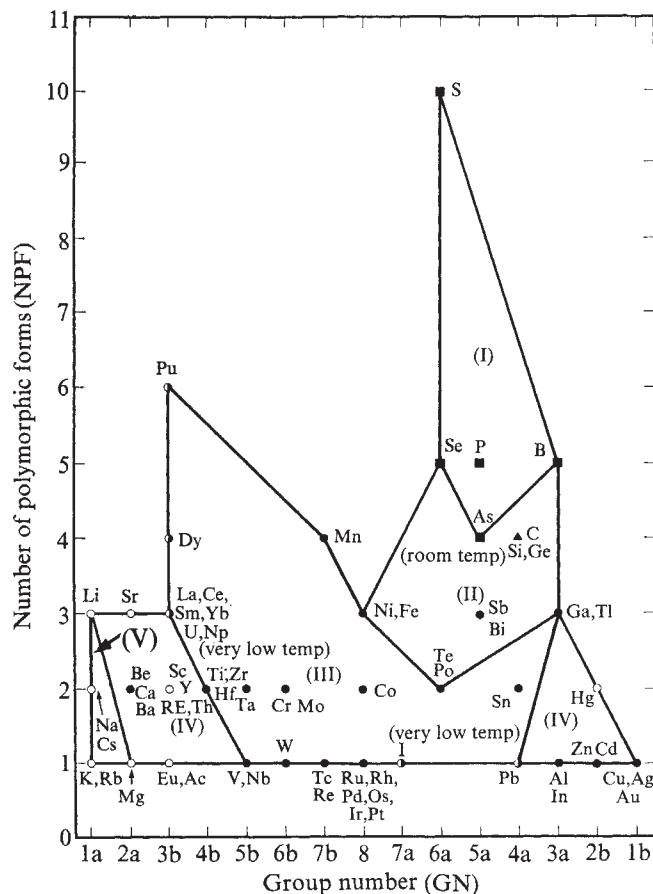


Fig. 1 Map showing the occurrence of elemental non-crystalline solids as correlated with the number of polymorphic forms (NPF) and group number (GN) of all the solid elements in the Periodic Table. The boundary lines are assigned to group elements conveniently into five regions representing the trend of formation conditions. The ease of formation of non-crystalline solids is illustrated by: ■, quenching from the liquid; ▲, formation by chemical or physical vapour deposition near room temperature; ●, formation by vapour deposition at low temperatures; half-filled circle, no data available, but we believe these can be formed in conditions similar to the ● data; and ○ no data available.

usually involves rapid quenching from high energy states (liquid or vapour) to prevent the forming of the lowest free-energy states. Instead, the atoms relax into a number of allowable bonding configurations separated by localised energy barriers. These allowable bonding types are those exhibited in the polymorphic structures. Therefore elements which have a large number of polymorphic forms will have a large number of available bonding states (energy minima) to accommodate the rapid relaxation process. The polymorphic bonding configurations would explain the low configurational entropy of non-crystalline phases as measured in Si and Ge³. The effect of GN on the occurrence of a non-crystalline phase can be understood in a similar context. Minimisation of the free energy by crystallisation becomes more unlikely as bonding anisotropy is increased from metallic bonds to covalent bonds, because of an increased 'jamming' probability.

Several recent observations support our concept of polymorphic bonding in non-crystalline solids. Structural models based on computer-simulated random packing give better agreement with diffraction results when variable bond lengths are allowed rather than single bond distances^{6,7}. The optical properties of amorphous silicon are more consistent with the bondings in the high pressure tetragonal polytype than with the diamond structure⁸. Moreover, the fact that there can be more than one 'polytype' for an amorphous

material, as in arsenic, in itself means that the non-crystalline state is characterised by specific bond types.

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An early Ordovician vertebrate

HETEROSTRACAN fishes are the oldest known fossil vertebrates, and well preserved examples are frequently found in rocks of late Silurian and Devonian age, but in older rocks their remains are fragmentary and rare. Their oldest occurrence yet described is in the Harding Sandstone, Colorado (middle Ordovician^{1,2}), where two genera occur; one of these has subsequently been recognised in marine limestones of similar age from Ontario, Canada³.

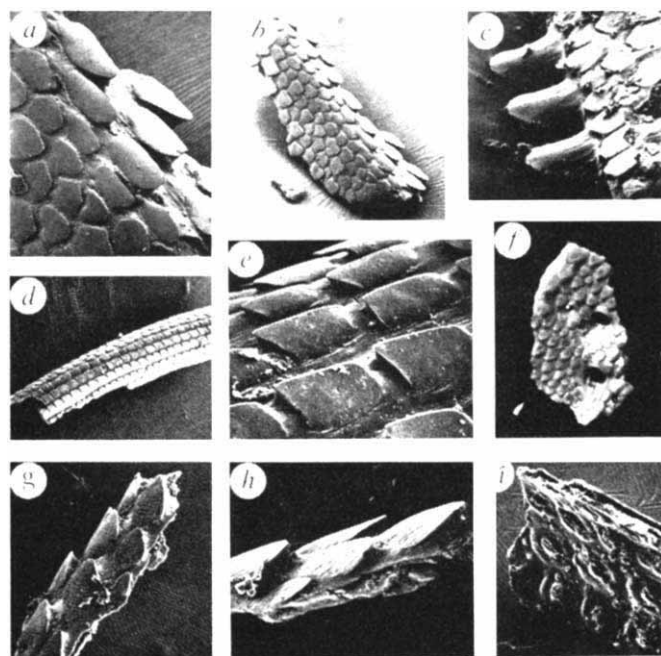


Fig. 1 Fragments of *Anatolepis heintzi* (a-f, i) and *Anatolepis* sp. (g and h) from the early Ordovician Valhallfonna Formation, northern Spitsbergen. f was taken with a light microscope, the others with a scanning electron microscope. a and b, Holotype, PMO NF 3263/1 ($\times 54$ and $\times 18$, respectively), showing typical scale types with smooth external surfaces; c, PMO NF 3263/2, part of spine-like fragment ($\times 36$), with transformed scales at edge; d and e, PMO NF 3263/3, elongate, U-shaped fragment with rhombohedral external scales ($\times 18$), and detail ($\times 180$); f, fragment with obliquely orientated, circular perforations, PMO NF 3263/4 ($\times 18$); g and h, PMO NF 3263/5, fragment of second species of *Anatolepis* with grooved scales; g, from above ($\times 30$); h, lateral view ($\times 94.5$); i, inner surface of a fragment, PMO NF 3263/6 ($\times 54$), showing low tubercles perforated at tip, and trilaminate structure at broken edge.

During the search for phosphatic microfossils from the early Ordovician Valhallfonna Formation, northern Ny Friesland, Spitsbergen¹¹, we recovered numerous small fossils which are identified as fragments of the earliest heterostracan, indicating that the earliest vertebrate occurrence predates that of the Harding Sandstone by about 20 Myr.

Whereas deposits containing later heterostracans were laid down in freshwater or brackish conditions⁴, the earliest sediments are regarded as fully marine, indicating that the vertebrates had their origin in the same medium as other major metazoan groups^{5,6}. The only recorded occurrence of vertebrate remains supposedly pre-Middle Ordovician are some small thelodont denticles—termed *Palaeodus* and *Archaeodus*—from the early Ordovician of the Russian platform⁷. These specimens are lost, and doubt has been cast on their authenticity (ref. 3 and S. Turner, personal communication). The Middle Cambrian problematicum *Eoichthys* Bryant^{8,9} is regarded as unlikely to be vertebrate; indeed it was later¹⁰ not considered as such by its original author.

The present finds were part of residues obtained by dissolving limestones in acetic acid, which otherwise include conodonts, chitinozoa¹², trilobites, brachiopods and radiolaria¹³. The associated fauna indicates a shallow shelf, fully marine environment, with a well oxygenated, soft, muddy sea floor, populated by a variety of benthic trilobites¹⁴. Vertebrate remains have been recovered from two horizons: 90–100 m from the base of the Olenidsletta Member and

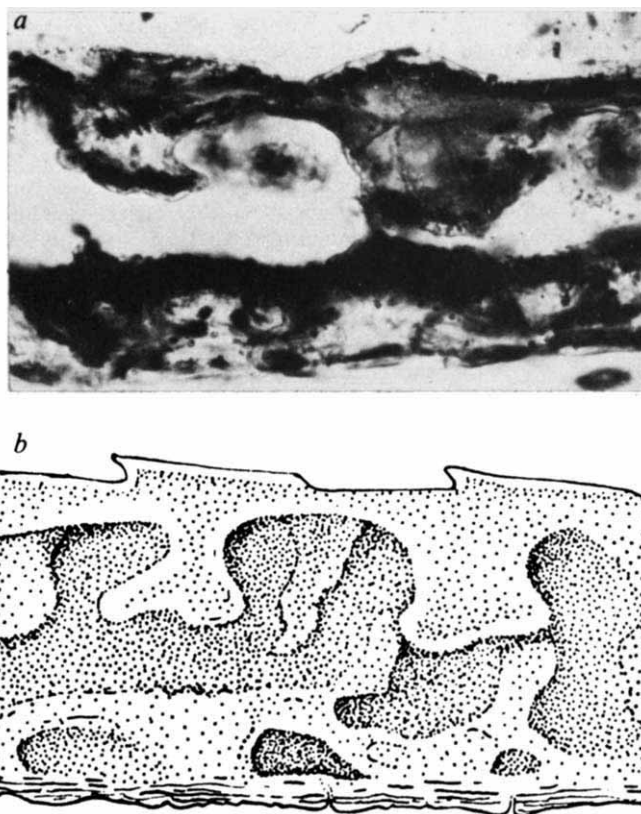


Fig. 3 *Anatolepis heintzi*. a, Photograph of thin section of PMO NF 3262 ($\times 750$), showing external scales, basal lamellar layer, intermediate cavernous aspidin layer. b, Generalised reconstruction of *Anatolepis* bone ($\times 750$).

30–50 m from the base of the Profilbekken Member. The former occurrence is with graptolites indicating an Arenig (early Ordovician zone of *Didymograptus hirundo*) age, the latter slightly younger, possibly earliest Llanvirn.

The specimens range in length from 1 to 2 mm, and one or more edges are invariably frayed, suggesting that the fragments are parts of larger plates. Analysis by X-ray diffraction shows that fragments are composed of apatite but the possibility of the hydroxyl (OH) radical being present (in small amounts) cannot be eliminated. The outer surfaces of the plates are covered with minute, smooth, elliptical to rhomboidal scales 25–150 μm long; the larger scales are concentrated at the edges of the fragments, where they are frequently more or less imbricated. Plates are frequently 'doubled over' at the edges with larger scales (Fig. 1a and b), such fragments possibly being from the perimeter of the animal (and if this is so, indicating a dorso-ventrally flattened overall shape). The inner surface of the plates carry tubercles of two sizes; the larger of these are perforated at their tips, these perforations presumably being the sites of dermal nerves or blood vessels (Fig. 1i).

Apart from flat or slightly curved fragments or 'edge' pieces, there are two particularly interesting fragments which, being covered with similar scales, are referable to the same animal. One is a spine (Fig. 1c) in which some of the scales are extended to form blades. The other fragment (Fig. 1f) includes small, circular openings, each equipped with a small lip externally and doubtless representing original openings in the exoskeleton. Since several groups of early agnathans had separate gill openings it is tempting to assume that these perforations represent the openings into the gill pouches, but they may also be pores into the lateral line system.

The plates are remarkably thin (70–100 μm) and delicate,

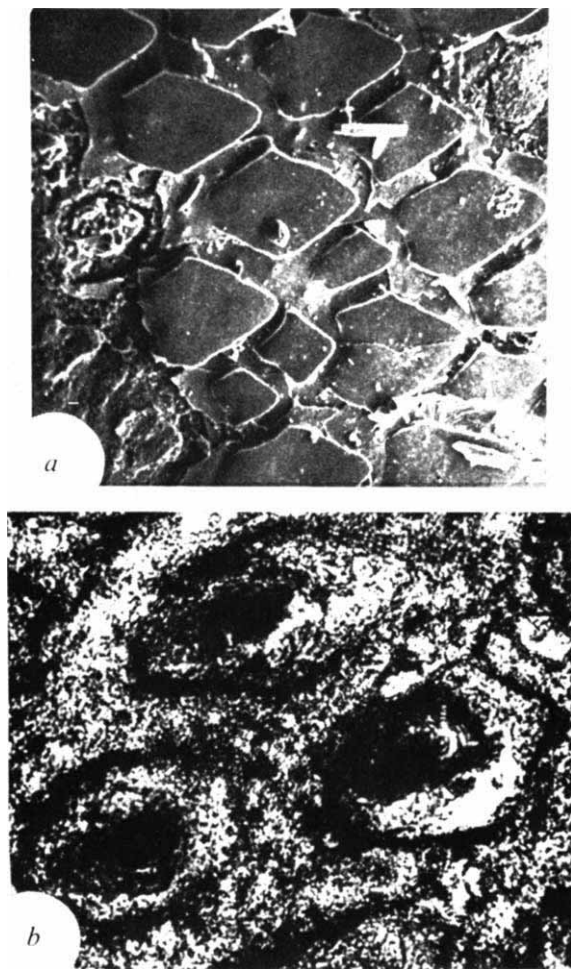


Fig. 2 *Anatolepis heintzi* a, PMO NF 3263/7 ($\times 300$), Scanning electron microscope photograph of part of fragment showing circular structures beneath each scale; b, PMO NF 3264 ($\times 1,600$), light microscope picture of similar fragment to a, photographed with transmitted light to show circular aspidin structures in middle of scales (NF 15, Fig. 10).

and this explains why larger fragments have not been recovered. In section the structure of the plates is similar to that described for other heterostracans^{15,16}, with a basal lamellar layer, an outer, probably dentinous, layer carrying the rhomboidal scales, the two separated by a spongy 'aspidin' layer (Fig. 1j, Figs 2 and 3): the latter easily tears during sectioning. Both *Astraspis* and *Eriptychius* from the Harding Sandstone are much more robust and have completely different external tubercles—the latter variously lobate, frequently with broad, subparallel raised ridges, the former with hemispherical, radially grooved tubercles. External tubercles of both these genera have more than twice the average diameter of those of the new material from Spitsbergen. Because of these differences we propose a new name for the species described here—*Anatolepis heintzi* gen. et sp. nov. The type species is named after the late Professor Anatol Heintz, who contributed much to the study of early fishes. A distinctive second species, with minutely grooved external scales (Fig. 1g and h), has been recovered (one specimen only) from the lower horizon.

More material, preferably still *in situ* on the bedding surfaces, is needed before an attempt at a reconstruction of the whole animal can be made. The fragments alone are of interest because they prove that heterostracans were already present in the earliest part of the Ordovician (500 Myr ago) and show that they were then certainly Marine. They also indicate that the thicker armour of the middle Ordovician forms is likely to have been secondary¹⁷. Material at hand shows no evidence of cyclomorial growth¹⁸ or fusion of smaller tesserae¹⁹, and it is possible that skeletisation occurred all at once in *Anatolepis* rather than by fusion of individual tubercles. If such fusion occurred it may have been in an ancestor of Tremadocian or Upper Cambrian age. Our discovery may stimulate examination of acid residues of pre-Ordovician age for the remains of still earlier vertebrates.

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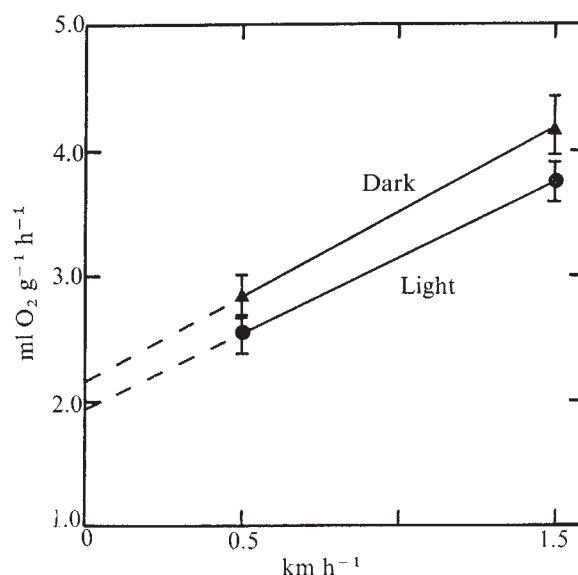
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synchronised with a higher basal metabolic rate as well as body temperature¹, and may also be reflected in metabolic needs for (the increased) locomotion occurring during the active period. Since the animal is using more energy simply to maintain its basic needs, there may also be a higher energy requirement for moving about. We have examined this, and have found that the increased basal rate persists as the constant difference (at different speeds), between the day and night performances of gerbils.

Three male gerbils (*Meriones unguiculatus*, weight 62–85 g; average 71.7 g) were purchased from local pet stores, and fed rat food and water *ad libitum* supplemented with seeds, fruits and vegetables. Between 20 and 26 h before each run their food was removed. The gerbils were placed on a 12-h light (0600–1800) and 12-h dark (1800–0600) cycle two weeks before the experiment. The animals were run under ordinary room light for the light runs and under dim red lights for the dark runs (the lighting conditions seemed to have no effect on their running ability). Running took place on a treadmill 40 cm long and 10.5 cm wide enclosed in a Lucite box (13×15×50 cm) kept at 20–22 °C, and the gerbil's speed was measured by timing revolutions with a stop-watch; it could be maintained to within $\pm 5\%$. Oxygen consumption was measured on a Beckman model F-3 oxygen analyser calibrated to within $\pm 0.005\%$ O₂ (1% of full scale deflection) by lowering the pressure within the measurement cell. Room air was pulled into the chamber at a rate of 226 l h⁻¹ (STP) $\pm 3\%$ giving a 75% turnover of the air in the chamber in 3.6 min (assuming complete mixing). The chamber was kept at a slight negative pressure, ensuring that all the air exhaled by the animals would be drawn into the oxygen analyser. After the gerbils had run for at least 20 min at each speed and were running well, data were taken for each 10-min period when the deflection of the oxygen analyser trace showed an oxygen variation of no more than $\pm 0.005\%$. Oxygen consumption was calculated by multiplying the air flow by the change in oxygen concentration. This value was then multiplied by 1.0425, on the assumption of a respiratory quotient (*R*) of 0.84, which would give a maximum $\pm 4.25\%$ error for *R* values of from 0.70–1.0 (ref. 6). In fact, the *R* value probably did not vary appreciably, since

Fig. 1 Oxygen consumption of gerbils running during their dark (▲) and light periods (●) at 0.5 and 1.5 km h⁻¹. Vertical bars with horizontal caps indicate ± 2 s.e.m.; there was no overlap of the means ± 2 s.e.m. The lines joining the means are extrapolated to the y intercept. The slope for the dark period running is 1.35 ml O₂ g⁻¹ km⁻¹ (s.e.m. = 0.140); for the light period it is 1.21 ml O₂ g⁻¹ km⁻¹ (s.e.m. = 0.107).



Do nocturnal rodents run more efficiently at night?

AN animal's activity is perhaps the most obvious physiological parameter influenced by circadian rhythms. Nocturnal animals move about and do their foraging during their hours of darkness. This higher level of activity is also

the animals were all in the same nutritional state during their runs.

For both running speeds used here (0.5 and 1.5 km h⁻¹) the oxygen consumption of animals running during their dark period was significantly higher than when they ran during their light period (Fig. 1). It takes more energy to run a given speed during the dark (active) period. The increments in oxygen consumption for the 1 km h⁻¹ difference in speed between 0.5 and 1.5 km h⁻¹ were, however, about the same, and were below those predicted for quadrupedal mammal of that size. The increment was 1.35 ml O₂ g⁻¹ h⁻¹ in the dark period, and 1.21 ml O₂ g⁻¹ h⁻¹ in the light period, compared with predicted value of 1.52 ml O₂ g⁻¹ h⁻¹ for a 72-g mammal². The y intercepts of the lines (2.17 and 1.94 ml O₂ g⁻¹ h⁻¹) are in good agreement with the predicted value of 2.04 ml O₂ g⁻¹ h⁻¹ (ref. 2).

The oxygen consumption data may be interpreted as if the resting requirement is higher during the dark period but the metabolic increase needed for running is the same during either period, in agreement with measurements of activity and metabolism in rats during dark and light periods³. Lizards and mammals of the same weight also show the same metabolic increments for running, even though the lizard has a much lower basal oxygen usage⁴.

Splitting the metabolic levels shown in Fig. 1 into a resting and a running component shows that the increment from running will be about the same whether the running occurs during the light or the dark period. Then, since the time of the day the animal chooses to run a given distance will affect neither the metabolic increment due to running nor the total resting oxygen consumption, the total oxygen consumption over a 24-h period will be the same. Whatever other advantages may accrue to the gerbils as nocturnal animals, such as safety from predators or decreased evaporative water loss during the cooler night periods⁵, there is no metabolic adaptation for more efficient running when the animals run the most.

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Increased incidence of triploidy in embryos derived from mouse eggs fertilised *in vitro*

FERTILISATION *in vitro* has now been achieved in several mammalian species, including rabbit¹, mouse², golden hamster³, rat⁴, and man⁵. The possibility exists that the technique may increase the incidence of chromosomal anomalies through any of several mechanisms including meiotic disturbance in the oocyte associated with superovulation, fertilisation by two or more sperm as a result of a high sperm concentration and/or a change in the natural block to polyspermy, altered conditions of competition between sperm with normal and abnormal genomes, and disjunctional irregularities during early cleavage of the fertilised egg. To determine whether any of these possible types of abnormality are of real significance will require extensive investigation. As yet the limited published data

from human^{6,7} and rabbit⁸ embryos are either too few to be useful or lack control observations. We have therefore made a cytogenetic study of the incidence of triploidy in mouse embryos following fertilisation *in vivo* and *in vitro* and have found a highly significant increase in triploidy in the *in vitro* group.

Care was taken in both series to arrange that the eggs fertilised were 'fresh' (that is, non-aged). In both groups, eggs were from 2–3 months old (C57BL/10×CBA) F₁ females with sperm provided by outbred TO males. All of the females were induced to superovulate with 5 IU pregnant mare's serum (PMS) (Gestyl, Organon), followed approximately 48 h later by 5 IU human chorionic gonadotropin (HCG) (Pregnyl, Organon). For fertilisation *in vivo* HCG was administered 12–13 h before the estimated time of natural ovulation. Females were paired with males and examined for vaginal plugs the following morning; the day of finding a plug was taken as day 1. For fertilisation *in vitro*, females were killed 13 h after HCG administration and their unfertilised eggs were immediately mixed with sperm. Thus, in both groups the eggs were the same approximate age at the time of association with sperm.

Table 1 Chromosomal analysis of mouse embryos derived from superovulated eggs fertilised *in vivo* and *in vitro*

	40	±40	±60	Triploidy (%)
<i>In vivo</i> days 3 and 4	162	17	5	2.7
<i>In vitro</i> 72-h culture	124	26	22	12.8

Experiments using the same *in vitro* system have indicated that sperm-egg union is complete within 1.5 h of gamete mixing (unpublished), a time which correlates well with *in vivo* studies indicating that sperm penetration can be detected within 1 h of mating^{9,10}.

Whittingham's medium based on Tyrode's solution¹¹ with 0.5 mM pyruvate was used for fertilisation *in vitro*. The medium for fertilisation contained 32 mg ml⁻¹ bovine serum albumin (BSA, Armour); that for culture contained 4 mg ml⁻¹ BSA. The preparation of the medium and dishes for experimental use followed the methods of Hoppe and Pitts¹². A sperm suspension was prepared by gently forcing the contents of two epididymides into the medium under paraffin oil with watchmakers forceps. After 20 min allowed for dispersal, a 1:9 dilution of the suspension giving a final concentration of 1.5×10⁶–3.0×10⁶ ml⁻¹ was used for fertilisation.

Eggs were released directly into the sperm and incubated at 37 °C for 3–6 h, then washed and cultured in droplets of medium under oil. After 72 h, embryos were at the 8–16-cell stage, and at that time the medium in the droplet was changed to one containing 10⁻⁸ M vinblastine sulphate (Velbe, Lilly) prepared in culture medium. Following incubation for a further minimum period of 3 h, chromosome preparations were obtained from the embryos.

In the experiments the mean *in vitro* fertilisation rate was 77.7% (two-cell/total eggs), and 89.6% of these two-cell embryos reached the 8–16-cell stage used for chromosomal examination. If cultured further, rather than being fixed, 80–90% of embryos would have been expected to form blastocysts, according to previous experience. Further, offspring of both sexes have been obtained after embryo transfer¹³.

Naturally mated females were injected on days 3 or 4 with 0.1 ml Velbe (1 mg ml⁻¹), and killed 2–5 h later to allow the recovery of embryos. The embryos of both *in vivo* and *in vitro* groups were placed into 1% sodium citrate for 8 min,

transferred to acid-cleaned slides, fixed rapidly with 3 drops 3:1 methanol-acetic acid and air dried (ref. 14, and E. P. Evans, personal communication). The preparations were stained with toluidine blue for cytogenetic analysis. Analysable preparations were obtained from 33% of the 528 embryos in the *in vitro* series and from 44% of the 414 embryos in the *in vivo* series.

The chromosomes were counted in all suitable blastomeres at mitotic metaphase (Table 1). The majority of counts were confidently either 40 or 60. No attempt was made to sex the embryos by identification of the Y chromosome. Some of the counts shown in Table 1 as ± 40 may have been genuine aneuploids but no instances of $2n/3n$ chimaerism or levels of polyploidy higher than $3n$ were identified. The difference between the frequencies of triploidy recorded in the *in vivo* series (2.7%) and the *in vitro* series (12.8%) is highly significant ($\chi^2=11.47$, $P<0.001$).

The mean frequency of triploidy among 3.5-d embryos derived from naturally ovulated eggs of 6 non-silver mouse strains was 0.9%, according to data given by Beatty¹⁵. The mean frequency in the exceptional silver strain was considerably greater, reaching 9.5% in one series. In an investigation of 1,359 preimplantation embryos from naturally ovulated (C3H $\times$ 101)F₁ females, 13 (1.0%) were triploid (E. P. Evans, personal communication). The latter embryos were obtained following morning matings and the eggs are therefore likely to have aged for several hours before fertilisation. Estimates of the frequency of triploidy among the preimplantation embryos of naturally mated, superovulated mice are 0.3% (ref. 16), 1.2% (ref. 17) and 1.8% (P. A. Neighbour, personal communication), in addition to our own figure of 2.7%. We have been unable to find an estimate of the contribution of superovulation itself to the incidence of triploidy in the mouse, which would require comparison with data from the embryos of matched, naturally ovulated and fertilised females.

Estimates ranging from 1.2% to 8.5% polyspermy in superovulated eggs fertilised *in vitro* have been derived from data obtained using Swiss mice^{18,19}. No control data from natural matings were provided so it is not possible to say anything about the significance of these figures. High frequencies of polyspermy have been recorded in superovulated eggs of the golden hamster fertilised *in vitro*, for example by Barros *et al.*²⁰, but such eggs have not been cultured past the two-cell stage²¹. All of these estimates were based on the number of pronuclei or sperm heads within the vitellus. Such observations provide no assurance that a synkaryon will result or that the egg will cleave. They cannot, therefore, have the same validity as counts of chromosomes at mitosis for the estimation of the proportion of resulting morulae that are triploid.

Counts of chromosomes have been reported in 26 human blastocysts obtained following *in vitro* fertilisation^{6,7}. All were said to have been diploid or approximately diploid. In view of the small number studied, these data are of limited value in excluding abnormalities of ploidy.

The explanation we favour for the striking rise in the incidence of triploid embryos following fertilisation *in vitro* is dispermy. This could be related either to a much higher sperm concentration *in vitro* than *in vivo*²² or to an alteration of the mechanism that normally prevents polyspermy. Disturbance at the second maturation division, leading to an increased frequency of digyny is also possible. The frequency of digynic triploidy, however, is unlikely to be great since the proportion of eggs with second polar bodies at the time of transfer to the culture medium is closely correlated with the proportion later observed to cleave (unpublished). A marker chromosome experiment to discriminate between these possibilities is in progress.

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Spread of antibiotic-resistant plasmids from chicken to chicken and from chicken to man

THE natural ecology of *Escherichia coli* and its infectious plasmids is not understood, although there is suggestive evidence that animals may serve as reservoirs for *E. coli* found in humans^{1,2}. Investigation in this area becomes additionally important in view of the practice of introducing plasmids with pieces of foreign DNA into *E. coli*^{3,4}. During an examination of the effects of antibiotic-supplemented animal feed on flora of farm animals and human personnel (ref. 5 and S.B.L., G.B.F. and A.B.M., unpublished), a study was initiated to determine if *R* plasmids and their *E. coli* hosts were naturally transferred among chickens and from chickens to human handlers. Our results illustrate the spread of antibiotic-resistant organisms from chicken to chicken and from chicken to man.

We used a temperature-sensitive chloramphenicol-resistance gene as a marker to identify a particular plasmid. *Escherichia coli* D1-7 (ref. 6), carrying R222, was treated with nitrosoguanidine⁷ and a mutant expressing temperature sensitivity to chloramphenicol was selected from the surviving bacteria. The temperature-sensitive chloramphenicol-resistance property was easily transferred to other hosts. This finding indicated its presence on a plasmid. This mutant plasmid, designated pSL222-6, expressed resistance to chloramphenicol, tetracycline, sulphonamides and streptomycin at 32 °C, but at 42 °C did not express chloramphenicol resistance. pSL222-6 was transferred by conjugation into two morphologically distinct *E. coli* isolated from chickens. An inoculum containing a mixture of the two recombinant bacteria was introduced directly into the intestines of four chickens by means of a tube placed in the cloaca. The chickens were caged separately and fed tetracycline-supplemented feed (Pfizer; tet-feed) to enhance colonisation with this tetracycline-resistant *E. coli*.

A repeat inoculation was carried out 4 d later although subsequent analysis showed that already at this time all

Table 1 Number of chickens excreting *E. coli* containing *pSL222-6**

Day	Cage			
	A	B	C	D
0	0	0	0	0
7	1	4	0	0
14	1	6	0	0
49	5	8	0	0
56	6	8	0	0
63	7	12	0	0
% Chickens excreting mutant <i>R</i> plasmid	14	24	0	0

*Does not include inoculated chickens placed in cages A and C; chickens in cages A and B were fed tet-feed; C and D received normal feed.

of the faecal *E. coli* isolated from these chickens were resistant to chloramphenicol at 32 but not at 42 °C. Two chickens were then placed in two cages each containing 50 chickens. One cage (A) was placed on the tetracycline-supplemented feed; the other (cage C) was not. Two other cages containing chickens with (B) and without tet-feed (D) were also maintained in the barn at about 50 feet from the experimental cages. In this way, the effect of tet-feed on the spread of the marked plasmid from inoculated chickens to other chickens could be evaluated.

Samples of faeces were taken weekly using a sterile swab (Handi-Swab, Fisher) from the cloaca and cultured on MacConkey agar plates at 30 °C. These master plates were then replica plated on to MacConkey agar containing chloramphenicol and incubated at 30 and 42 °C. Table 1 illustrates the spread of the mutant plasmid from the initial two chickens to the rest of the animals in these cages. After 2 months, *E. coli* with temperature-sensitive chloramphenicol resistance were detected in 14% of chickens in cage A. Of particular note was the finding of *E. coli* with this particular *R* plasmid in faeces of 24% of chickens in a distant cage (B). None of the bacterial samples from chickens in cages not placed on tet-feed (C and D) demonstrated the mutant *R* plasmid.

Since only the plasmid was marked in this first experiment, a second study was carried out to determine whether the plasmid was being spread by way of the host bacterium. Plasmid *pSL222-6* was introduced into a mutant strain of chicken *E. coli* resistant to nalidixic acid (*Nal^R*). (*Nal* resistance is not normally found among *E. coli* in chicken gut flora.) The *Nal^R* *E. coli*-bearing *pSL222-6* were introduced by cloacal inoculation into four chickens previously fed tet-feed. These chickens were placed in a cage of 18 chickens also eating tet-feed and excreting organisms resistant to tetracycline, and the presence of the doubly marked *E. coli* followed (Table 2). After 1 week, 7 of the 18 chickens were excreting *Nal^R* *E. coli*, all of which showed temperature-sensitive chloramphenicol resistance. By 5 weeks these *E. coli* were detected in all the chickens in the cage. Spread was more rapid than in the previously described experiment. About 20–30% of *E. coli* in the flora of these animals was *Nal^R* (*pSL222-6*).

Over a two-month period faeces from eleven family members living on the farm and three laboratory workers

responsible for the sampling of the chickens were tested for the presence of *pSL222-6*. On two occasions the plasmid was detected in human faeces. The first occurred in the faecal sample from the 12-yr-old son of the farm owner; 2 d after he had cleaned the cage in which chickens harbouring *E. coli* with this plasmid were housed, 78% of the coliforms in his faecal flora were resistant to chloramphenicol and this resistance was temperature sensitive. The *E. coli* also showed resistance to streptomycin and sulphonamides, but not to tetracycline. These resistances were not transferable. A similar non-transferable tetracycline-sensitive segregant of *pSL222-6* was found in *E. coli* from chickens in the cleaned cage. The presence of a plasmid in this *E. coli* was verified by the identification of a fast-moving peak of labelled DNA in an alkaline sucrose gradient (data not shown). Furthermore, other plasmids of the same incompatibility group (FII) were unstable when conjugated into this *E. coli*. This finding indicated the presence of a plasmid of the same incompatibility type as *pSL222-6*. Since typing of *E. coli* was not possible and the *E. coli* were not marked we could not determine whether the organisms were of human or chicken origin.

The second human host observed excreting *E. coli* with *pSL222-6* was an animal handler. One week after she had sampled chickens inoculated with the *Nal^R* *E. coli*-bearing *pSL222-6*, her faecal flora showed the presence of temperature-sensitive chloramphenicol-resistant *E. coli*. These *E. coli* were also resistant to nalidixic acid. All three other resistances on *pSL222-6* were also present. In conjugation the resistances in this *E. coli* were transferred together.

Subsequent faecal specimens from these two individuals 3 and 5 d after the initial sample did not reveal the presence of these particular organisms. Neither individual was taking antibiotics. Since only faecal swabs were examined, further studies would be necessary to determine whether these organisms were present to small extents in their intestinal tracts.

Our results demonstrate the spread of resistant organisms from chicken to chicken and from chicken to man. In studies of 1-d-old chicks, Smith⁸ found *E. coli* from one group of chickens in faecal samples from a second group of chickens within 2 d of their being mixed in a cage. In the present study spread of the mutant *R* plasmid and *Nal^R* *E. coli* was detected in adult chickens in the same and distant cages. The mechanism of spread, whether by air or on the clothes of the feed handler, is not known. Cage-to-cage spread of bacteria is probably a common occurrence in a farm environment, followed by subsequent spread of plasmids and bacteria among the chickens in the cage.

In the light of our findings, transfer may also occur among other animals and between other animals and man. In the absence of direct contact and of selective pressure, such as that exerted by antibiotics, the frequency of spread is probably small. The subsequent transfer of animal *R* plasmids to human *E. coli* in the intestine, however, could occur⁹ especially if the recipients were taking an antibiotic related to resistance determined by an *R* plasmid¹⁰. Although as few as 10⁴ organisms can produce colonisation of the human intestinal tract¹¹ most studies *in vivo* have indicated that more than 10⁶ bacteria need to be ingested^{12,13}. This seems too many to have been ingested without their

Table 2 Spread of newly introduced *R⁺* *E. coli* among chickens

	Week				
	0	1	3	4	5
Number of new chickens excreting <i>Nal^R</i> <i>E. coli</i> (<i>pSL222-6</i>)	4	7	3	2	2
Accumulated number of chickens excreting <i>Nal^R</i> <i>E. coli</i> (<i>pSL222-6</i>)	4	11	14	16	18

Four chickens excreting *Nal^R* *E. coli* bearing temperature-sensitive chloramphenicol-resistant *R* plasmid *pSL222-6* were introduced into a cage of 18 chickens previously fed tetracycline and excreting tetracycline-resistant *E. coli*. Spread of *Nal^R* *E. coli* was followed by replica plating initial faecal samples, from MacConkey agar plates, on to (1) MacConkey agar + chloramphenicol at 30 and 42 °C; (2) MacConkey agar + nalidixic acid (30 µg ml⁻¹) at 37 °C. All *Nal^R* colonies were temperature sensitive for chloramphenicol and vice versa.

knowledge by the two individuals in this study. The results suggest a different route of entry or a different culture medium for the ingested bacteria, for example, by way of the nasal passages to sputum to the gastrointestinal tract.

These data bear on the widespread use of antibiotics in veterinary and medical practice and on the proposed use of *E. coli* as a vehicle in genetic engineering experiments. Further studies on the conditions affecting transfer and mixing between animal and human bacteria will be important in evaluating the consequence of antibiotic-resistant bacteria in animals in contact with man.

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Thermostability studies for investigating non-electrophoretic polymorphic alleles in *Drosophila melanogaster*

ON theoretical grounds it is generally believed that most structural genes have common isoelectrophoretic alleles^{1–3}—polymorphic alleles that cannot be discovered by electrophoresis—but there is no direct evidence for this. Methods that might be used to search for such structural variability (involving, for example, thermostability or kinetic studies) have not often been adopted as screening procedures though they have been used to compare already identified electrophoretic alleles. This is not only because such methods are often unsuitable for screening purposes. Even when a screening method can be applied, its efficiency in revealing

existing structural variability is not known *a priori*, not even approximately, as for electrophoresis. Moreover, this unknown efficiency is presumably very low because although electrophoresis, being a separation technique, separates the products of two alleles in heterozygotes, other screening procedures only pick up the combined products which may or may not show an intermediate characteristic. It would usually be extremely difficult to distinguish between the three types. Thus, for any enzyme for which such a method is available, a two-step procedure seems convenient: (a) to test it on already known structural (electrophoretic) alleles of that enzyme, and if it turns out that it is efficient (b) to use the method in a search for structural isoelectrophoretic alleles. It must be assumed that these two types of structural differences are, on average, equally likely to affect the parameter being studied.

Drosophila melanogaster provides convenient material for such an approach because it is easy to synthesise isogenic lines for whatever gene has been mapped. In this way all relevant comparisons can be made between homozygotes for the alleles being studied. Phosphoglucosylase (PGM) depends in *D. melanogaster* on a single autosomal gene (3: 43.6) (ref. 4) with two common, *Pgm*^A and *Pgm*^B, and several rare electrophoretic alleles, *Pgm*^C, *Pgm*^D, *Pgm*^E and *Pgm*^F (ref. 5). Therefore this gene is quite suitable for this purpose. Thermostability is relatively simple to study, even for homogenates of a single fly. Two procedures were used to evaluate the thermostability of the various allelic PGMs studied: (1) incubation of two aliquots of a homogenate of several *Pgm* isogenic flies at two temperatures followed by electrophoretic analysis and (2) incubation at two temperatures of two slices of a starch gel after electrophoresis of an homogenate of a single fly followed by the addition of the PGM-staining mixture⁶. Temperature and time of incubation were decided empirically.

Figure 1 shows that our technique enabled us to classify any PGM band as heat resistant (R) or heat sensitive (S) on an alternative basis. Moreover, since heterozygous individuals were found in which the product of one *Pgm* allele was resistant and the product of the other *Pgm* allele was sensitive, we conclude that this property depends on the *Pgm* structural gene.

Presumably, there were available for heat-sensitivity studies several possible rare *Pgm* alleles because, besides those variants which are electrophoretically different, variants with the same electrophoretic behaviour but different in origin—if they are rare—are likely to be structurally different. Table 1 shows a large heterogeneity as regards heat sensitivity between both electrophoretically different *Pgm* alleles and rare alleles with the same electrophoretic behaviour but different origins. Moreover, as it might be expected, the latter heterogeneity was not found within the same population. It therefore seems reasonable to compute as certainly or probably distinct alleles any two *Pgm* rare variant alleles differing from each other in electrophoretic behaviour or origin, respectively. Of 16 such variant alleles 10 turned out to be R (5 *Pgm*^C and 5 *Pgm*^D) and 6 S (2 *Pgm*^E, 3 *Pgm*^F and 1 *Pgm*^G). Conclusive

Table 1 Classification of several rare *Pgm* variant alleles based both on their electrophoretic and heat-sensitivity behaviour

Origin of the strains	Electrophoretic Heat sensitivity	PGM phenotypes							
		C		D		E		F	
		R	S	R	S	R	S	R	S
Laboratory		1	—	2	—	—	1	—	—
Puglia	Castellaneta	2	—	1	—	—	—	—	1
	Otranto	2	—	—	—	—	—	—	—
	Corato	1	—	—	—	—	—	—	—
Sicilia	Ranna	—	1	6	—	—	1	—	—
	Archi	1	—	4	—	—	—	—	—
	Vittoria	—	1	1	—	—	2	—	—

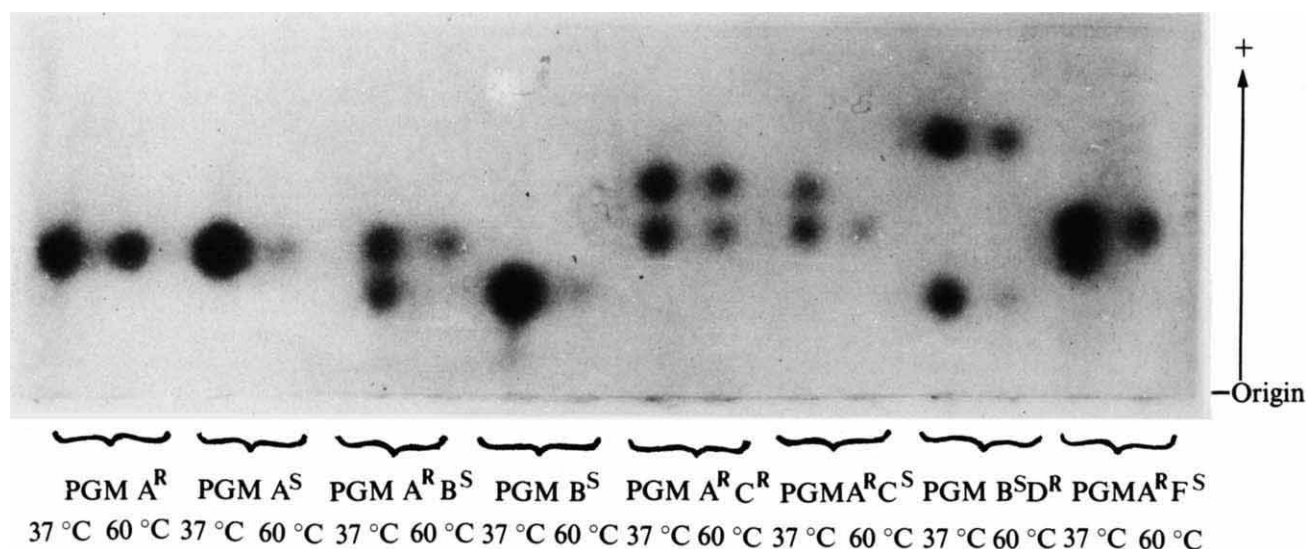


Fig. 1 Heat-sensitivity behaviour of various *Pgm* alleles. In the first procedure, for each strain to be examined 50 adult flies were homogenised in 1 ml of Tris-maleate buffer, pH 7.4, 10^{-3} M, diluted with 4 ml of the same buffer, centrifuged at $3,000g$ for 15 min and the clear supernatant subdivided into two aliquots which were incubated in a waterbath for 15 min, one at 37°C and the other at 60°C . Both were then analysed by the standard electrophoretic technique⁶. Every strain was classified on the basis of four replicated experiments. In the second procedure, for each fly to be analysed the homogenate (40 μl) was electrophoresed by the usual method. At the end of the electrophoretic run the starch gel was cut into two slices, which were transferred into thin plastic envelopes. One was dipped for 15 min in a waterbath at 37°C , the other at 60°C and then stained. By both these methods a given electrophoretic PGM band was classified in a clear cut way as a heat-resistant band (PGM R) or as a heat-sensitive band (PGM S) according to whether or not its PGM activity was detected after the treatment at 60°C .

evidence that the heat-sensitivity behaviour of each PGM electrophoretic band depends on the corresponding *Pgm* structural allele is presented in Tables 2 (first procedure) and 3 (second procedure). It seems that the phenotype for heat sensitivity of each PGM band travels with the electrophoretic phenotype of the same band and is restricted to it (*cis-trans* effect). Therefore its genetic site(s) must be at the *Pgm* structural gene.

It was thus possible to search for a further polymorphic heterogeneity of the *Pgm* structural gene besides the electrophoretic one. For this purpose we determined the heat-sensitivity phenotype of common electrophoretic *Pgm* alleles derived from a laboratory and two natural populations. These alleles were tested, by the first procedure, in either the homozygous or heterozygous state as isogenic or TM2 balanced lethal stocks, respectively (Table 4). The

previously homogeneous *Pgm*^A allele was split into two common isoelectrophoretic alleles, *Pgm*^{A, tr} and *Pgm*^{A, ts} in all samples examined. The *Pgm*^B allele seems so far to be homogeneous. Therefore a new type of structural heterogeneity of the *Pgm* gene has been discovered. This increases considerably the estimate of the degree of identified variability of this gene. For example, the degree of heterozygosity of the Castellaneta population increases from 0.17 based on the electrophoretic variation only (*Pgm*^A: 0.906; *Pgm*^B: 0.083; all the *Pgm* rare alleles: 0.011)⁵ to 0.30.

Bernstein *et al.*⁷ and Singh *et al.*⁸ have reported common isoelectrophoretic alleles of xanthine dehydrogenase in 11 species of the virilis group of *Drosophila* and octanol dehydrogenase in *D. pseudoobscura* with different thermostabilities. But they have not shown (1) that the heat-sensitivity phenotype is different in different allelic products of

Table 2 Testing the hypothesis that the heat-sensitivity of the PGM bands depends on alleles at the *Pgm* locus

Strains	Crosses	♂	♀	F ₁ Ubx phenotypes	F ₁ electrophoretic phenotypes† at 37 °C	at 60 °C
166 × 63		A ^S × B ^S			AB	—
68 × 63		A ^R × B ^S			AB	A—
103 × 55		A ^R × B ^S			AB	A—
55 × 103		B ^S × A ^R			AB	A—
138 × 63		A ^S × B ^S			AB	—
63 × 138		B ^S × A ^S			AB	—
246* × 63		D ^R /A ^R × B ^S		{ Ubx + 232	AB	A—
				{ Ubx 192	DB	D—
11* × 63		C ^R /A ^R × B ^S		{ Ubx + 215	AB	A—
				{ Ubx 241	CB	C—
63 × 11*		B ^S × C ^R /A ^R		{ Ubx + 180	AB	A—
				{ Ubx 218	CB	C—
57* × 63		E ^S /A ^R × B ^S		{ Ubx + 215	AB	A—
				{ Ubx 178	EB	—
126* × 63		C ^S /A ^R × B ^S		{ Ubx + 203	AB	A—
				{ Ubx 174	CB	—
63 × 126*		B ^S × C ^S /A ^R		{ Ubx + 195	AB	A—
				{ Ubx 213	CB	—
132* × 5		A ^S /A ^R × B ^S		{ Ubx + 245	AB	A—
				{ Ubx 216	AB	—

*Balancer lethal stock; the TM2 third balancer chromosome carries simultaneously the ultrabitorax (Ubx¹³⁰) morphological dominant mutation and the *Pgm*^A heat-resistant allele.

†Determined on 50 adult-fly homogenates according to the first procedure described in the legend of Fig. 1.

Table 4 Distribution of common *Pgm* alleles identified by combining electrophoretic and heat sensitivity determinations

Origin of the strains	Total genes examined*	Classification of the <i>Pgm</i> genes examined†			
		<i>Pgm</i> ^{A, tr}	<i>Pgm</i> ^{A, ts}	<i>Pgm</i> ^{B, tr}	<i>Pgm</i> ^{B, ts}
Laboratory	18	12	3	—	3
Castellaneta (Puglia)	61	45	4	—	12
Ranna (Sicilia)	67	45	2	—	20

Estimates of gene frequencies (obtained by multiplying the relative frequencies of heat-resistant and heat-sensitive alleles in each electrophoretic class by the gene frequencies of the corresponding electrophoretic alleles):

$$\begin{array}{lcl}
 \text{Castellaneta} & & \text{Ranna} \\
 \left. \begin{array}{l} Pgm^{A, tr} = p_1 = 0.832 \\ Pgm^{A, ts} = p_2 = 0.074 \\ Pgm^{B, ts} = q = 0.083 \\ Pgm \text{ rare} = r = 0.011 \end{array} \right\} & P = 0.906 & \left. \begin{array}{l} p_1 = 0.907 \\ p_2 = 0.040 \\ q = 0.041 \\ r = 0.012 \end{array} \right\} P = 0.947
 \end{array}$$

p, *q* and *r* are the estimates previously obtained by considering the electrophoretic phenotypes only⁵. *p* has been split further into *p*₁ and *p*₂ according to the relative frequencies of *Pgm*^{A, tr} and *Pgm*^{A, ts} alleles.

*Each of these genes was tested either as an isogenic line homozygous for that gene or as a balanced lethal stock in which that gene was heterozygous with a *TM2* chromosome carrying a *Pgm* allele with a known electrophoretic and heat-sensitivity phenotype.

†We propose to indicate the heat sensitivity of the *Pgm* electrophoretic alleles or phenotypes by adding to their name a superscript, *tr* and *ts* corresponding to thermoresistance and thermosensitivity, respectively.

Table 3 Absolute association between the Mendelian segregation of the electrophoretic and heat sensitivity phenotypes of four *Pgm* alleles

F ₁ genotypes	Type of cross $\frac{A^R}{B^S} \times \frac{C^S}{D^R}$	F ₁ electrophoretic phenotypes*	
	No. of observed individuals	at 37 °C	at 60 °C
A ^R C ^S	95	AC	A—
A ^R D ^R	76	AD	AD
B ^S C ^S	95	BC	—
B ^S D ^R	83	BD	—D

*Determined in single-fly homogenates following the second procedure described in the legend of Fig. 1.

the structural gene studied in appropriate heterozygotes, and, as a necessary consequence of this, (2) that the heat-sensitivity phenotype is transmitted together with a certainly identified structural phenotype (for example, electrophoretic). Therefore their evidence seems to be incomplete because it has not been excluded that the observed heat sensitivity phenotypes were due to a different gene. For example, the thermostability of the autosomal enzyme adeninephosphoribosyltransferase, in man is greatly influenced by the sex-linked hypoxanthineguaninephosphoribosyltransferase (HGPRT) deficiency⁹. Nevertheless it seems likely that those alleles exist because situations such as HGPRT deficiency are probably not common and, on a theoretical basis, many structural genes are expected to have polymorphic isoelectrophoretic alleles.

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Nude mice from homozygous nude parents show smaller PFC responses to sheep erythrocytes than nude mice from heterozygous mothers

THE congenitally athymic mouse which is homozygous for nude (*nu/nu*), compared with the phenotypically normal heterozygote (*+/nu*) or the normal mouse (*+/+*) is severely deficient in its plaque-forming cell (PFC) response to sheep erythrocytes (SE)^{1,2}. The minimal response of the nude is generally attributed to a deficiency of thymus-derived helper cells which are essential for a full PFC response to thymus-dependent antigens such as SE^{1–3}. The amount of helper activity (T cell or T-cell-like) that nude mice may receive from their mothers is of concern in several laboratories^{4–9}, since most nude mice obtained routinely are born of phenotypically normal (*+/nu*) mothers that have T cells. There is debate about the relative importance of T-cell precursors observed in nude mice¹⁰, which may be stimulated to express T-cell antigens by soluble factors¹¹, and the equally important possibilities that nudes may receive T cells and/or soluble factors *in utero* from their normal *+/nu* mothers or from their *+/nu* or *+/+* littermates or from the colostrum of *+/nu* mothers¹². The results of studies designed to resolve this issue remain equivocal^{4–13}. There is a need¹² for studies with nude mice born of homozygous nude parents (*nu/nu* ♀ × *nu/nu* ♂) and raised by nude mothers to minimise the possibilities of transfer of functionally mature T cells *in utero* or the transfer of T cells or T-cell-like factors by the colostrum. So far this criterion has been difficult to meet since nude mothers from most common mouse strains were poorly fertile or could not raise their young¹⁴.

In initial experiments using NIH strain nude mice, we compared the 4-d PFC responses of nude mice of the following parentages, nude (*nu/nu*) mothers × nude (*nu/nu*) fathers (type I), heterozygous (*+/nu*) mothers × nude fathers (type II), and type I offspring, foster nursed by *+/nu* mothers (type III). The breeding and husbandry of these nude offspring is to be detailed elsewhere. Briefly, the NIH nude mouse colony was established in the summer of 1972. The initial stock was received on a BALB/c background. The *nu* gene was next established into the N: NIH(S) outbred stock of mice, which has a very high reproductive performance. Breeding at this stage was conventional, that is, heterozygous (*nu/+*) females with homozygous (*nu/nu*) males yielding 50% homozygous offspring. In the second stage the *nu/nu* progeny was established and maintained in a specific pathogen-free (SPF)

Table 1 PFC responses of NIH strain nude mice from nude mothers and heterozygous mothers to sheep erythrocytes

Type of nude		Type of mothers	Nursed by	Cells per spleen ($\times 10^6$)	PFC per 10^6 cells	PFC per spleen
I	Mean $\pm$ s.e. for 10 mice	nu/nu	nu/nu	126 $\pm$ 7	1.4 $\pm$ 0.09	174 $\pm$ 11
II	Mean $\pm$ s.e. for 10 mice	+ /nu	+ /nu	197 $\pm$ 16	12.8 $\pm$ 3	2,652 $\pm$ 701
III	Mean $\pm$ s.e. for 6 mice	nu/nu	+ /nu	153 $\pm$ 9	6.1 $\pm$ 0.5	932 $\pm$ 105

Plaque-forming cell (PFC) responses to SE of type I nude mice (homozygous nude mothers) and of type II nude mice (heterozygous +/nu, phenotypically normal mother) and type III (homozygous nude mothers, but foster nursed by +/nu mothers) are compared. The fathers in all cases were homozygous nudes. Each mouse was injected intravenously with 4×10^6 SE and the direct PFC count per spleen or per 10^6 acid-resistant splenocytes was estimated for individual mice by the localised haemolysis-in-gel assay described before^{15,16}. Cells per spleen were estimated electronically in the Fisher Autocytometer-II. Thymus tissue was not visible in nude mice of this study.

Table 2 *In vitro* PFC responses to sheep erythrocytes by splenocytes from NIH strain nude mice and the effect of dibutyryl cyclic AMP

Type of nude splenocytes cultured	Dibutyryl cyclic AMP	Splenocytes recovered after 4 d in culture ($\times 10^6$)	PFC detected after 4 d of culture per 10^6 recovered splenocytes	PFC per culture
I Mean $\pm$ s.e. for 10 serial cultures	—	10.6 $\pm$ 0.3	3.7 $\pm$ 1.8	42 $\pm$ 6
I Mean $\pm$ s.e. for 5 serial cultures	+	12.5 $\pm$ 0.8	32.6 $\pm$ 5.6	417 $\pm$ 95
II Mean $\pm$ s.e. for 10 serial cultures	—	9.6 $\pm$ 0.4	16.5 $\pm$ 2.5	153 $\pm$ 17
II Mean $\pm$ s.e. for 5 serial cultures	+	12.6 $\pm$ 3.2	24.2 $\pm$ 1.6	371 $\pm$ 17

Comparison of *in vitro* plaque-forming cell (PFC) responses of dispersed spleen cells from type I nude mice and type II nude mice to sheep erythrocytes (SE) after 4 d in culture. The effect of dibutyryl cyclic AMP (1.5 μ M per culture of N^6 , O²-dibutyryl adenosine-3'-5'-cyclic phosphate, Calbiochem) on similar cultures are compared. Type I splenocytes (31×10^6 per culture) and type II splenocytes (29×10^6 per culture) were cultured in the presence of 2×10^6 SE as antigen in modified Marbrook chambers as described before¹⁶. The number of cells and the number of PFC were estimated as stated in the legend for Table 1.

environment by hysterectomy and foster nursing on previously established germ-free (GF) mice. This environment consists of an SPF open room barrier facility, requiring that all supplies which enter be sterile. Movement of personnel who enter is closely controlled and personnel wear sterile garments, including face masks and gloves. The rooms and animals are monitored for infectious bacteria and viruses. No evidence of infectious viruses has been found. This includes scrutiny for mouse hepatitis virus (MHV), to which nude mice are extremely susceptible. In these conditions, survival of homozygotes to 28 months was achieved. In the third stage, 30 pairs of homozygous matings were established as soon as animals were available. It was important to scrutinise the colony continuously and to separate individuals suggestive of sterility and lactation-failure. By these procedures, the total number of homozygotes weaned from homozygous mothers is not much smaller than that for heterozygous mothers. Our experience is that homozygous mothers weaned an average of 9.2 offspring during their reproductive life compared with an average of 12 by heterozygous mothers.

The PFC responses of type I nudes (PFC/spleen) were 15 times less than those of type II nudes (Table 1). The difference estimated on a per cell basis (per 10^6 cells) was approximately tenfold. Type III nudes, which were produced by foster nursing type I nudes on T-cell positive, +/nu mothers showed PFC responses that were four to five times greater than type I nudes (Table 1).

In spleen cell cultures (Table 2), type I nude mouse PFC responses were similarly less than those of type II nudes. Dibutyryl cyclic AMP which exerts a T-cell like helper effect¹⁵ augmented tenfold the PFC culture responses of type I spleen cells, raising them to approximately the control level of type II cultures not exposed to the compound.

These data show that nude mice from homozygous nude parents are more immunologically deficient with respect

to their ability to activate antibody-forming cells than the more conventionally bred nude mice derived from heterozygous mothers. The greater PFC responses observed in nude mice borne by nude mothers, but nursed from birth by heterozygous foster mothers are consistent with the suggestion that athymic nude mice receive T-cell help from heterozygous mothers. Further clarification of the possible roles of both T cells and soluble T-cell-like factors awaits isolation and identification of the cells and factors. The status of T-cell precursors in nude mice described here remains to be evaluated.

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Suppression of adoptive antibody responses by addition of spleen cells from agammaglobulinaemic chickens "immunised" with histocompatible bursa cells

THE report that T cells from patients with common variable hypogammaglobulinaemia can suppress immunoglobulin synthesis by B cells *in vitro*¹ implies that some forms of hypogammaglobulinaemia could, at least in part, be maintained by a patient's own T cells. Blaese *et al.*² described a phenomenon of "infectious agammaglobulinaemia" in line 6 chickens (USDA Regional Poultry Research Laboratory, East Lansing) which seems to represent an ideal laboratory model for the human disease. Transfer of agammaglobulinaemia with spleen cells has now also been observed consistently in strain FP and SC chickens²². The mechanism whereby recipients of cells from an agammaglobulinaemic donor are rendered agammaglobulinaemic is, however, difficult to analyse in such a long term *in vivo* system. Thus, further examination of the phenomenon would be greatly facilitated by a short term assay to allow better identification of the target cell.

We describe here a transfer system in which the suppressor activity of spleen cells from agammaglobulinaemic chickens on the adoptive immune response of normal spleen or bursa cells is assayed in neonatal irradiated recipients. The target system used is the antibody response to *Brucella abortus* (BA), which is thymus independent³ and readily detected in recipients within 1 week of bursa cell transfer⁴. Using this 1-week assay, we have found that spleen cells from bursectomised (BX) donors interfere with an adoptive anti-BA response only when they are taken from BX animals previously injected with bursa cells.

Donor and recipient birds were of strains FP (B15/B21) and SC (B2/B2) (Hy-Line International). BX birds were obtained by *in ovo* injection of 3.75 mg testosterone propionate (Elkins-Sinn) on day 11 of gestation, followed by intraperitoneal injection of 4 mg cyclophosphamide (Mead-Johnson) on the day of hatching, and 3 mg on each of the 2 subsequent days⁵. Sera of BX birds were tested for Ig by double diffusion in agar, using rabbit antiserum to chicken Ig to detect readily Ig at 1% normal 8-week serum level.

Some BX birds of the FP strain were injected intravenously with 2×10^7 male bursa cells 6–13 d before being killed. For the SC strain, the injection schedule was more varied (Table 3). Chicken Ig (mixture of IgM and IgG,

Table 2 Effect of pretreatment of suppressor cells on inhibition of adoptive immune response, FP strain

Bursa-immune BX spleen (4×10^7)*	Pretreatment of BX spleen†	Mean log ₂ agglutinin serum titres in recipients $\pm$ s.e.‡	
		Day 7	Day 14
—		14.4 $\pm$ 0.8	14.0 $\pm$ 0.9§
+	None	0.3 $\pm$ 0.0	4.9 $\pm$ 2.1
+	Mitomycin	4.1 $\pm$ 0.4	8.3 $\pm$ 2.0
+	NRS+C	0.0 $\pm$ 0.0	2.8 $\pm$ 2.0
+	Anti-T+C	11.8 $\pm$ 1.3	11.5 $\pm$ 1.7§

*On day 0, recipients injected intravenously with 4×10^7 bursa cells from 6-week-old donors, 2×10^8 BA, with or without 4×10^7 viable spleen cells from a BX donor; this BX donor received 2×10^7 bursa cells intravenously on day -13 (experiment 4).

†Mitomycin ($50 \mu\text{g ml}^{-1}$) 15 min at 37 °C; anti-T or normal rabbit serum (1/90) and C (1/18) 37 °C for 1 h.

‡Anti-BA agglutinin titres determined on sera taken on day 7; recipients were reinjected with BA on day 7 and bled on day 14.

§0.2 < P < 0.3.

prepared as described⁶) was injected subcutaneously with Freund's complete adjuvant (FCA).

Recipients were either injected with 4 mg of cyclophosphamide intraperitoneally on the day of hatching and treated with 500 r. whole-body ¹³⁷Cs γ irradiation (Gammator M. Radiation Machine Co.) or they received 500 and 800 r. on days -2 and -1, respectively. On the day of transfer, day 0, donor cell suspensions were prepared in 0.165 M phosphate-buffered saline containing 0.2% bovine serum albumin and, in the case of bursa cell suspensions, heparin ($2 \mu\text{g ml}^{-1}$). BX spleen cells (6.7×10^6 cells per ml) were treated with rabbit anti-T serum⁷ or normal rabbit serum (NRS) (1/90) and young guinea pig complement (C) (1/18) for 1 h at 37 °C. Treatment with mitomycin was for 15 min at 37 °C at 10^7 cells per ml. BX spleen donor cells (4×10^7 viable) and target bursa or spleen cells (4×10^7) were mixed just before injection into leg veins of recipients (six to eight per group). The BA antigen was also added immediately before injection (approximately 2×10^8 washed dead bacteria). Anti-BA agglutinin titres were determined on sera taken 1 week after transfer, and in some cases on sera taken at 2 weeks after a second BA injection on day 7. The day-14 sera (minimum of four chickens per group) were also analysed after exposure to 0.1 M mercaptoethanol in order to measure 7S antibody titres⁸. Experiments lasting 2 weeks were possible as control animals injected with antigen alone on days 0 and 7 gave no appreciable responses.

The results in Table 1, obtained in the FP strain, show

Table 1 Ability of spleen cells from BX chickens to inhibit adoptive antibody production by bursa or normal spleen cells, FP strain

Responding cell type (4×10^7)	BX spleen added* (4×10^7)	BX donor injection†	Mean log ₂ agglutinin serum titres in recipients $\pm$ s.e.‡		
			Expt 1 Day 7	Expt 2 Day 7	Expt 3 Day 7
Spleen	—		5.0 $\pm$ 0.9	5.3 $\pm$ 1.7	
Spleen	+	Nothing	5.3 $\pm$ 0.8	5.4 $\pm$ 1.2	
Spleen	+	Bursa	1.6 $\pm$ 0.5	2.3 $\pm$ 0.6	
Spleen	+	Ig		5.8 $\pm$ 1.1	
Bursa	—			11.3 $\pm$ 0.3	12.0 $\pm$ 0.7
Bursa	+	Nothing		10.9 $\pm$ 0.5	10.8 $\pm$ 0.9§¶
Bursa	+	Bursa		1.6 $\pm$ 0.8	5.0 $\pm$ 0.3§¶
Bursa	+	Spleen			8.7 $\pm$ 0.2
Bursa	+	Thymus			9.5 $\pm$ 0.9¶
Bursa	+	Ig		10.3 $\pm$ 0.3	

*On day 0, recipients injected intravenously with responder cells from 6–9-week-old donors, BA (2×10^8), with or without spleen cells from agammaglobulinaemic chickens (3–4 months old).

†"Immunisation" of BX donors with bursa, spleen or thymus cells injected intravenously as follows: experiments 1 and 2, 2×10^7 cells on day -6 or -7; experiment 3, 2×10^6 cells on day -13. "Immunisation" with chicken Ig was on day -7, 200 μg with CFA injected subcutaneously.

‡Anti-BA agglutinin titres determined on sera taken on day 7.

§P < 0.001.

¶0.3 < P < 0.4.

Table 3 Ability of spleen cells from BX chickens to inhibit adoptive antibody production by bursa cells, SC strain

Cell type added (4×10^7)*	BX donor injection†	Mean log ₂ agglutinin serum titres in recipients $\pm$ s.e.‡				
		Expt 5 Day 7	Expt 6 Day 7	Expt 7 Day 7	Expt 8 Day 7	Expt 8 Day 14
None		9.8 $\pm$ 0.4	14.2 $\pm$ 0.3	9.3 $\pm$ 2.1	11.7 $\pm$ 0.8§	15.8 $\pm$ 2.0 (7.3 $\pm$ 1.1)¶
BX Spleen	Nothing	11.5 $\pm$ 0.3	14.3 $\pm$ 1.2			
BX Spleen	Bursa, once	10.5 $\pm$ 0.2	13.5 $\pm$ 0.4	4.8 $\pm$ 0.4	10.4 $\pm$ 1.0	9.8 $\pm$ 2.1 (2.8 $\pm$ 0.9)
BX Spleen	Spleen	10.7 $\pm$ 0.5				7.7 $\pm$ 2.0 (3.0 $\pm$ 0.6)¶
BX Spleen	Bursa, twice				6.5 $\pm$ 0.7§	

*On day 0, recipients injected intravenously with bursa cells from 6–9-week-old donors and BA (2×10^6), with or without spleen cells from agammaglobulinaemic chickens (3–4 months old).

†“Immunisation” of BX donors with bursa or spleen cells administered as follows: experiment 5, 2×10^7 cells intravenously day –7; experiment 6, 2×10^7 cells intravenously on day –21; experiment 7, 2×10^7 cells intravenously on day –89; experiment 8, 2×10^7 cells with CFA subcutaneously on day –7 (“once”) and 2×10^7 cells intravenously on day –52 (“twice”).

‡Anti-BA agglutinin titres determined on sera taken on days 7 and 14.

§ $P < 0.001$.

¶Titres remaining after incubation of sera with 2-mercaptoethanol are in parentheses. $P < 0.01$.

that BX spleen, injected together with either bursa or spleen cells from 6–12-week-old normal chickens, did not inhibit responsiveness of normal cells. But when spleen cells were taken 1–2 weeks after intravenous injection of 2×10^7 bursa cells into 3–4-month-old BX chickens, they caused a considerable reduction of the adoptive antibody response (Tables 1 and 2). Injection of fewer bursa cells (2×10^6 , experiment 3) was somewhat less effective but still seemed to immunise the BX donors much better than did 2×10^6 spleen or thymus cells. The lack of immunisation with thymus cells suggested that bursa cell-specific antigen rather than antigen determined by minor histocompatibility loci was responsible for the immunisation effect. The age of the BX chicken at which immunisation with bursa cells could be effected was not determined precisely, but in one experiment (not in Tables 1 and 2) such an immunisation proved ineffective in a 2-month-old BX chicken. Other attempts to immunise BX chickens were made using chicken-Ig in CFA; these had no effect (experiment 2, Table 1). This suggested that Ig molecules, as present in serum, did not constitute the moiety against which BX spleen cells were reacting. The fact that the inhibitory effect on bursa and spleen cells occurred in recipients which obviously had circulating maternal IgG (ref. 9) supports this conclusion. Further efforts towards identification of the bursa cell antigen included an experiment (not in Tables 1 and 2) in which immunisation with 4×10^6 paraformaldehyde-treated (30 min at 4°C , 5×10^6 cells and 0.1 g ml^{-1}) bursa cells failed.

Since bursa cells from young chickens generally give a higher adoptive response to BA than do spleen cells⁴, experiments to characterise the effector cell further were performed with bursa cells as the target. “Bursa-immune”, BX spleen cells still reduced the immune response after exposure to mitomycin ($P < 0.05$ compared with $P < 0.01$ for untreated cells, Table 2). In addition, the effector cell in the BX spleen was largely eliminated by treatment with rabbit anti-T but not by NRS+C, suggesting its T-cell nature (Table 2). Thus, the BX spleen cell responsible for this phenomenon seems to be a T cell which, after recent immunisation with its target cell, does not need to proliferate to inhibit B cells. For prolonged maintenance of B-cell suppression in recipients, however, the untreated or NRS-treated BX spleen cells were more effective than were the mitomycin-treated (compare day 14 with day 7 results, Table 2).

Several of the recipients in experiment 4 which showed no anti-BA on day 14 after transfer of bursa cells + “bursa-immune” BX spleen still failed to produce anti-BA 4 weeks later and had no detectable Ig in their sera. This latter observation suggests strongly that the phenomenon studied

here is analogous to that described as “infectious agammaglobulinaemia” by Blaese *et al.*² and Palladino *et al.*²².

As mitomycin-treated cells were effective, an experiment was performed in which a 5 and a 9-month-old FP chicken—birds which were undergoing and which had undergone complete involution of the bursa—were injected with bursa cells and used as spleen donors 1 week later. We reasoned that natural involution of the bursa might be caused by an immune response to bursa antigens occurring at a later age. Mitomycin was required since adult spleen cells would otherwise transfer their own anti-BA response. The mitomycin-treated cells from these spleens did not reduce the ability of bursa cells to give adoptive anti-BA responses. This approach, therefore, was discontinued.

Results with the SC strain were much less striking than those with the FP strain (Table 3). Immunisation with neither bursa nor with spleen cells caused a rapid appearance of bursa-inhibitory activity in the spleen cells of agammaglobulinaemic donors. A longer interval after immunisation with bursa cells may have been needed, as suggested by the results of experiments 7 and 8. The later (day 14) bleedings in experiment 8 indicated that both 19S and 7S anti-BA antibody formation were suppressed. There was also a decreased response in a later bleeding taken in experiment 6 (not shown in Table 3). In this experiment, the day 14 data were very similar to those in experiment 8, but loss of several control recipients precluded appropriate analysis.

Experiments by Palladino *et al.*²² have also shown that transfer of agammaglobulinaemia was obtained more readily in FP strain than in SC strain, although inhibition of antibody formation could be demonstrated in both. It was further observed in our laboratory that FP chickens give far better delayed hypersensitivity reactions to protein antigens than do SC chickens, suggesting a much more responsive T-cell system in the FP strain. The results reported here suggest that the difference between the FP and SC strains noted by Palladino *et al.* was due to a variation in T-cell reactivity and not to a difference in B-cell mass, since a uniform dose of target cells was used in our experiments. The more readily demonstrable BX spleen immunity to bursa cells in the FP strain may also be related to the relative ease in the FP compared with the SC strain with which agammaglobulinaemia can be obtained (unpublished).

The antigen to which the splenic T cells of BX chickens respond is unknown. Candidates include (1) Ig in a form peculiar to the B-cell surface (IgD analogue)¹⁰; (2) viral antigen which might normally be expressed on bursa and B cells only^{11,12}, and (3) any other surface antigen unique to B cells^{7,13} in the resting and/or activated state. This is

reminiscent of the conclusion of Jacobson *et al.*¹⁴ that the target antigen on cells in long term, T-cell mediated¹⁵ allotype suppression could not be Ig1b in the same form as it is in the circulation. This phenomenon in mice, applying to a subpopulation of B cells, seems strikingly similar to that of transferred agammaglobulinaemia in chickens, where all B-cell function becomes suppressed. It has also been suggested that suppressor cells have a role in the maintenance of long term allotype suppression of rabbits¹⁶.

Since the suppression of antibody production in our studies as well as in those of Palladino *et al.*²² and Blaese *et al.*² results in agammaglobulinaemia of recipients, the phenomenon is not likely to be antigen specific. Tada and Takemori¹⁷, however, have noted that even in studies on antigen-specific T-cell suppression of the immune response, the susceptibility of B cells to the specific suppressive influence seems to differ depending on the stage of differentiation of B cells and/or on the class of Ig they synthesise.

The lack of tolerance to an organ which was removed during ontogeny and reintroduced later in development was described originally for the hypophysis in tadpoles¹⁸. The observation that immunity to bursa cells can be induced in chickens deprived of bursa early in ontogeny may similarly be ascribed to a lack of tolerance to tissue (bursa)-specific antigens. The development of immune T cells, in this case called suppressor cells, may then represent the normal mechanism of cytotoxic cell development such as is observed to minor transplantation antigens¹⁹ and to tumour antigens^{20,21}.

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Modification of fibrosarcomas and squamous cell carcinomas of mouse skin using various tissue extracts

RECENT attention has focused on the possibility of utilising (or mimicking) aspects of mitotic processes in the control of cancer. Bullough, Rytömaa and others used preparations of mitosis inhibiting proteins (chalones) to produce remissions (total in some cases) in a number of tumour tissues¹⁻³. These chalone preparations were shown to be tissue specific. Only epidermal chalone worked on epidermal tumours. Liver extracts prepared in the manner of epidermal chalone extracts gave no remissions in epidermal tumours⁴. Melanocyte chalone was necessary for remissions in melanomata⁵ and granulocyte chalone was successful in the treatment of chloroleukaemia⁶. We report here remissions resulting from the treatment of chemically-induced fibrosarcomas and squamous cell carcinomas of the skin in mice using extracts of various mouse tissues. We also report the presence of mitosis-inhibiting protein in these extracts.

Excised whole skin, liver or tumour, frozen in liquid N₂ and minced, was homogenised with 0.14 M NaCl-0.14 M phenol in a Waring Blender for 15 h at 4 °C. After centrifugation for 2 h at 2,500 r.p.m., the supernatant, combined with 5% sodium dodecyl sulphate in 45% EtOH, was stirred for 24 h at 4 °C, then centrifuged (1 h, 2,500 r.p.m.). The resulting supernatant was transferred to a new tube and again centrifuged (1 h, 2,500 r.p.m.). The final supernatant was combined at 4 °C with an equal volume of cold 95% EtOH and the precipitate collected.

One portion of the extract obtained from whole skin was treated with α -chymotrypsin to remove protein. A second portion was treated with deoxyribonuclease and ribonuclease to remove nucleic acids. Analysis, using modified Dische techniques⁷, of the extracts showed that the percentage DNA tripled after chymotrypsin treatment relative to the untreated skin extract and, after deoxyribonuclease-ribonuclease treatment, was one-sixth that of the untreated skin extract. Each of the five extracts (the three tissue extracts and the two treated skin extracts) were suspended in sterile 0.9% saline (100 μ g extract per 0.05 ml) for injection.

Fibrosarcomas and squamous cell carcinomas were induced in male BALB/c mice through three injections each

Table 1 Percentage remissions for all animals

Group	No. of mice	Percentage remissions		
		1 month*	2 months*	3 months*
(1) Treated with unreacted skin extract	28	53	†	†
(2) Treated with unreacted skin extract	40	48	28	5
(3) Treated with liver extract	19	47	16	21
(4) Treated with skin extract reacted with chymotrypsin	14	21	29	17
(5) Treated with skin extract reacted with nucleases	18	56	17	17
(6) Treated with tumour extract	12	0	0	33

*Percentage of each group still in remission 1, 2, or 3 months after treatment was started.

†Tumour size measurements not made.

Table 2 Percentage remissions for animals with squamous cell carcinomas

Group*	No. of mice	Percentage remissions		
		1 month†	2 months†	3 months†
(1)	15	73	‡	‡
(2)	16	88	56	50
(3)	12	67	25	8
(4)	6	50	67	50
(5)	8	75	25	0
(6)	7	0	0	57

*Groups are the same as in Table 1.

†Percentage of each group still in remission 1, 2 or 3 months after treatment was started.

‡Tumour size measurements not made.

of 100 µg (in 0.05 ml linseed oil) of either 9,10-dimethylbenzanthracene or benz(a)pyrene spaced over a period of 2 weeks. Tumours were developed for 3 months and then the animals were screened. Those with very large tumours (larger than about 10 cm³) and those showing no tumour development were excluded (in the environmental control group no evidence of spontaneous tumour formation was observed). Tumours were then inoculated with 5–100 µg injections (over a 1-month period) of one of the extracts. Effects of the extracts on tumours were monitored by size measurements. Selected animals were killed and tissue samples examined by the Department of Pathology, College of Medicine, University of Kentucky.

Untreated fibrosarcomas and squamous cell carcinomas showed no spontaneous remissions. Injection into tumours of extract or of 0.9% saline produced uniform minimal mechanical damage. The injection of 0.9% saline alone produced no remissions. Subcutaneous injection of tumour extract or of skin extract into healthy animals produced no apparent effects, establishing the absence of any general cytotoxic material in those extracts.

In two separate experiments, with separate preparations of the whole (untreated) skin extract and with separate animal populations similar effects on tumours were noted (Tables 1, 2 and 3). For all animals in these groups (both those with fibrosarcomas (FS) and those with squamous cell carcinomas (SCC)) the percentage of animals showing remissions one month after initiation of treatment with skin extract were 53% (*n*=28) and 48% (*n*=40). For animals with SCC, the percentage remissions were 73% (*n*=15) and 88% (*n*=16). For animals with fibrosarcomas, the percentage remissions were 31% (*n*=13) and 21% (*n*=24). The smaller percentages observed for animals with FS could result from the difficulty in reaching all parts of the tumour with the extract. FS were larger than the SCC, and may have been too large to treat in some cases—sizes at treatment initiation ranged up to 10 cm³. The percentages fall after the first month, suggesting that the effect of the skin extract may be temporary and/or that a longer period of treatment may be necessary. The animals with continuing remissions had smaller tumours initially.

Table 3 Percentage remissions for animals with fibrosarcomas

Group*	No. of mice	Percentage remissions		
		1 month	2 months	3 months
(1)	13	31	‡	‡
(2)	24	21	8	0
(3)	7	14	0	0
(4)	8	0	0	0
(5)	10	25	10	10
(6)	5	0	0	0

*Groups as in Table 1.

†Percentage of each group still in remission 1, 2 and 3 months after treatment was started.

‡Tumour size measurements not made.

The liver extract produced remissions in 47% (*n*=19) of all mice treated with liver extract (Table 1). The liver extract produced remissions in 67% (*n*=12) (Table 2) of the animals with SCC and 47% (*n*=7) (Table 3) of the animals with FS. If the extraction of skin and liver yield the same products, then these percentages for the liver extracts, which are similar to those for the untreated skin extract, indicate a lack of tissue specificity in the extract actions. Tumour extract produced delayed remissions only (0% 1 and 2 months after treatment initiation for both FS and SCC; 33% (*n*=12) for all animals 3 months after treatment; 57% (*n*=7) 3 months after for SCC; and 0% (*n*=5) after 3 months for FS) (Tables 1, 2, 3). The reason for these delayed remissions is uncertain, but it may be that the remission-producing protein in the tumour extract is present in a decreased amount.

Treatment of tumours with skin extract reacted with nucleases produced remissions in percentages (56% (*n*=18) for all animals; 75% (*n*=8) for SCC; 25% (*n*=10) for FS) (Tables 1, 2, 3) similar to those observed following treatment with the unreacted skin extract. Skin extract treated with chymotrypsin, however, yielded markedly reduced remission percentages (21% (*n*=14) for all animals; 50% (*n*=6) for SCC; 0% (*n*=8) (Tables 1, 2, 3), suggesting that the active component in the skin extract is protein.

An experiment measuring the uptake of ³H-thymidine into cultures of mouse ear in Hank's medium demonstrated the presence of a mitosis-inhibiting substance in untreated skin extract. This material may or may not be the same material which affects tumours.

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Mediation of retinoic acid-induced growth and anti-tumour activity

VITAMIN A (retinol) is a nutrient essential for vision, growth, reproduction and proper differentiation of epithelial tissue¹. Some retinol is oxidised to retinoic acid (vitamin A acid) *in vivo*², but if an animal is provided with retinoic acid in place of dietary retinol, it can only partially substitute for the missing retinol; vision³ and reproduction are impaired⁴. Sporn *et al.*⁵ have described two systems for evaluating the activity of retinol, retinoic acid, and their analogues *in vitro*. One measures the ability of such compounds to promote growth by determining RNA, DNA and protein levels after addition of the test compound to epidermal cell cultures derived from newborn mouse skin; the other measures ability to reverse metaplasia of the epithelial cells of trachea explanted to organ culture from vitamin A-deficient hamsters. Retinoic acid is active in both systems.

In addition, retinoic acid and an aromatic analogue of the acid have been shown to reverse metaplasias of mouse skin induced by application of carcinogenic drugs and expressed as papillomas and tumours⁶. The molecular mechanism(s) by which retinoic acid or retinol control

growth and differentiation, as well as the mechanism of their carcinostatic ability, is still unknown but may involve the specific cytoplasmic binding proteins we have discovered for retinol and retinoic acid. The cellular retinol-binding protein binds retinol *in vitro*⁷ and *in vivo*⁸, is present in many tissues, and exhibits binding affinities for *cis* isomers of retinol which parallel the growth-promoting activity of these isomers in the whole animal⁹. The cellular retinoic acid-binding protein binds retinoic acid *in vitro* with high specificity and is present in normal rat⁸ and human organs¹⁰ as well as some human cancers¹¹. The two proteins have been separated physically and purified 1,000-fold from rat testis⁸. There has also been a preliminary report of a retinoic acid-binding protein in embryonic chick skin¹².

In our investigations into the mechanism(s) of action of retinol and retinoic acid we have examined newborn mouse skin, hamster trachea, and chemically induced papillomas of mouse skin for the presence of the cellular retinoic acid-binding protein. In addition, using extracts of mouse skin papillomas, human breast tumour, and a 1,000-fold purified preparation from rat testis as sources for the binding protein, we have examined its relative affinity for some ring analogues of retinoic acid (Fig. 1). In all cases we find that the binding characteristics of the protein towards the analogues correlate with the activity of the compounds in the assay systems of Sporn *et al.* as well as with reported tumour-inhibiting activity.

Newborn mouse skin, adult mouse skin, mouse skin papillomas induced by treatment with 7,12-dimethylbenz(a)anthracene and croton oil⁶, hamster trachea, and human breast tumour were homogenised in 0.05 M Tris-HCl, pH 7.1, and soluble extracts prepared as described previously⁸. A preparation of cellular retinoic acid-binding protein, purified 1,000-fold from rat testis as described before, was also used⁸.

The binding protein was detected and its affinity to various analogues was determined as described earlier, using radioactive retinoic acid and sucrose gradient centrifugation⁸. The binding affinity was assessed by competition experiments and expressed as percentage inhibition (percentage decrease) of binding of radioactive all-*trans* retinoic acid observed when the competing analogue was present in a 100-fold molar excess over the ³H-retinoic acid⁸. Details of the determination are presented in Table 1.

Analysis of extracts of newborn mouse skin and of hamster trachea revealed a peak of radioactivity in the 2S region of the sucrose gradient, indicating the presence of cellular retinoic acid-binding protein. It was also established that the mouse skin papillomas have a high level of the binding protein, quantitated as described previously⁸, (860 pmol of ³H-retinoic acid bound per g wet tissue) compared with control skin of mice of comparative age (56 pmol ³H-retinoic acid bound per g of wet tissue). This

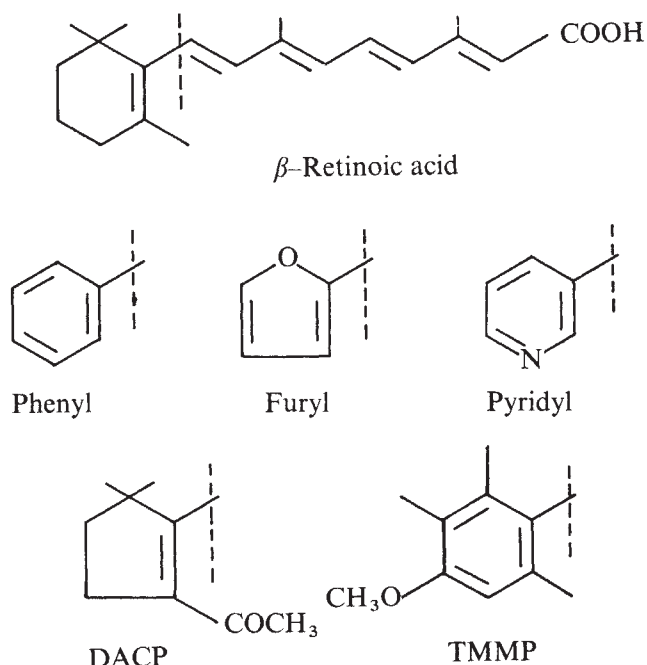


Fig. 1 Structures of β-retinoic acid and analogues with ring modifications.

difference may be due to an increase in the numbers of a cell type which normally contains this binding protein, or due to an altered expression of the genome. We have also found that several malignant human tumours (breast and lung) contained the cellular retinoic acid-binding protein but it was not detectable in normal tissue from the same organ of the same patient¹¹.

The presence of this binding protein suggests that the protein might mediate the action of retinoic acid. This contention is strengthened by the affinity which the cellular retinoic acid protein exhibits, independent of the source, towards analogues of retinoic acid. Both the dimethylacetylcyclopentenyl (DACP) and trimethyl-methoxyphenyl (TMMP) analogues of retinoic acid are effective competitors for the binding of retinoic acid (Table 1). The phenyl, furyl and pyridyl analogues were less effective competitors in the order listed, with presumably decreasing affinities. These results correlate well with the ability of these compounds to promote growth in epidermal cell cultures derived from mouse skin and to reverse the metaplasia of hamster trachea induced by vitamin A deficiency as shown by Sporn *et al.*³. These authors noted that DACP and TMMP had marked activity in their systems, whereas the phenyl analogue had minimal activity and the furyl and

Table 1 Percentage inhibition of binding of all-*trans* ³H-retinoic acid to cellular retinoic acid-binding protein by analogues of retinoic acid

Compound	Rat testis binding protein (%)	Mouse papilloma binding protein (%)	Human breast tumour binding protein (%)
All- <i>trans</i> retinoic acid	100	100	100
DACP analogue	100	100	100
TMMP analogue	100	100	100
Phenyl analogue	42	36	36
Furyl analogue	34	16	24
Pyridyl analogue	22	0	0

The preparations, made to 0.3 ml with 0.05 M Tris, pH 7.1, were incubated with 1×10^{-7} mmol radioactive all-*trans* retinoic acid ($1.37 \text{ Ci mmol}^{-1}$) in the absence and presence of 1×10^{-8} mmol unlabelled retinoic acid analogues shown in Fig. 1. Each compound was added in 5 μl ethanol containing $1 \text{ mg } \pm\alpha\text{-tocopherol ml}^{-1}$. Incubation mixtures whose protein concentration was below 1 mg ml^{-1} also contained 0.3 mg myoglobin and 0.3 mg purified bovine serum γ -globulin fraction. After incubation in the dark at 4 °C for 5 h, 0.2 ml of a charcoal-dextran solution was added with agitation. After 5 min the charcoal-dextran was removed by centrifugation and 0.2 ml of the supernatant liquid was layered on a 5–20% linear sucrose gradient and centrifuged at 148,000g for 20 h. The gradients were collected in twenty fractions and the radioactivity of each fraction determined. Binding to the cellular retinoic acid-binding protein was indicated by a peak of radioactivity in the 2S region of the gradient.

pyridyl analogues seemed to be devoid of activity, compared with retinoic acid.

The apparent affinity of the cellular retinoic acid protein towards the TMMP analogue is of particular interest in that administration of the ethyl ester of this compound, or retinoic acid itself, is effective in preventing induction of mouse skin papillomas. These compounds can also cause the disappearance of the papillomas once induced⁸.

The DACP analogue of retinoic acid is reported to block the ability of 3-methylcholanthrene to induce hyper- and metaplasia of prostate epithelium¹³. We have not examined this tissue for presence of the binding protein but we note, as have others¹², that the DACP analogue binds effectively to the protein.

It has been suggested that the reversal of metaplasias induced by vitamin A deficiency and by chemical carcinogens by retinoic acid and its TMMP analogue may be similar phenomena¹⁴. The striking similarity between the binding affinity of the cellular retinoic acid protein for retinoic acid analogues and the growth-promoting as well as anti-tumour activity of these analogues suggests, circumstantially, that the action of retinoic acid in these instances could be mediated by this cellular protein.

Furthermore, the results reported here, as well as those reported earlier for cellular retinol binding protein⁹, suggest that evaluating analogues of retinol and retinoic acid for their ability to bind to cellular retinoic acid or retinol-binding protein may be a useful preliminary screening step to identify those with potential growth or anti-tumour activity. Conversely, screening tumours for the presence of binding protein may indicate possible responsiveness of the tumours to treatment by such compounds.

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different media depleted in certain low molecular weight nutrients reversibly ceased growth in either the G₁/G₀ phase (phosphate, histidine plus glutamine deprivation) or at random (methionine, leucine) in the cell cycle^{9,10}. We have found that insulin and other agents stimulated these cells (except those arrested by leucine depletion) to synthesise DNA and divide, whereas fibroblast growth factor (FGF) which normally initiates cell growth in quiescent cultures arrested predominantly by serum depletion of the medium^{5,6} was ineffective. This suggests a possible differential basis for their normal mode of action.

Swiss mouse 3T3-4A fibroblasts in medium containing 10% serum cease growing mainly because of the exhaustion of serum growth factors. Addition of low concentrations (50 ng ml⁻¹) of FGF initiated approximately 40% of these cells to synthesise DNA whereas addition of similar concentrations (50 ng ml⁻¹; Table 1, 250 ng ml⁻¹; not shown) of insulin alone yielded only a small increase. When added with FGF, however, insulin substantially increased the number of cells synthesising DNA, in agreement with previous reports^{11,13}. Addition of the original concentration of phosphate (Table 1), glucose or amino acids¹⁴ (not shown) had no effect. This showed that the cultures were completely dependent on the addition of macromolecular components in serum for the reinitiation of cell growth.

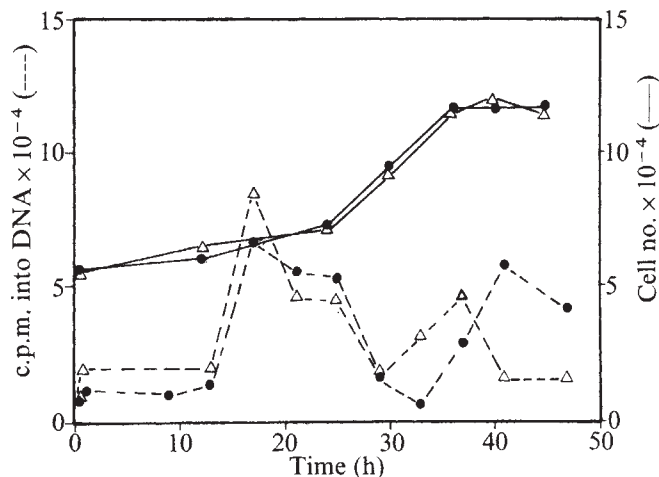


Fig. 1 Time of induction of DNA synthesis and cell division. Cultures were plated in medium containing 1/100 of the normal phosphate concentration and grown as in Table 1(2). After 3 d insulin (100 ng ml⁻¹) (●) or normal concentrations of phosphate (Δ) in DEM was added and cultures were exposed for 3 h to ³H-thymidine (3 μCi ml⁻¹) at 3 μM after varying times. The indicated time represents the mid-point of the 3-h exposure to ³H-thymidine (---). The c.p.m. recorded represent two-thirds of the total radioactivity incorporated into DNA per culture. Cell numbers per Petri dish were also recorded (—).

Nutrient-dependent arrest of fibroblast growth is partially reversed by insulin but not fibroblast growth factor

REINITIATION of the growth of quiescent, non-transformed fibroblasts in tissue culture is achieved by addition of serum^{1,2} or low concentrations of certain polypeptide hormones³⁻⁶. These agents which are thought to interact primarily with the surface of the cell⁴ presumably require a second signal to be transmitted to the interior. An increase in the intracellular "availability" of low molecular weight nutrients has been suggested to fulfil such a role^{7,8}. Mouse fibroblasts placed in

Non-growing 3T3-4A cultures were also obtained by reducing the phosphate, glutamine plus histidine, methionine or leucine concentrations to respectively 1/100, 1/25, 1/25, 1/100 of that present in normal medium. Complete removal of any of these essential nutrients led to cell death within 24 h. Addition of FGF, at 50 ng ml⁻¹ (Table 1) or up to 250 ng ml⁻¹ (not shown) barely increased the number of radioactively labelled nuclei or the number of cells. Addition of hydrocortisone with FGF was also without effect (Table 1). Addition of 250 ng ml⁻¹ of insulin alone, however, initiated substantial increases in DNA synthesis (50-60% radioactively labelled nuclei) and in cell numbers in cultures starved for phosphate and histidine plus glutamine (Table 1). Insulin's growth-promoting effect occurred at concentrations from 10 ng ml⁻¹ reaching a maximum at about 100 ng ml⁻¹ (not shown), while the time required to induce DNA synthesis and cell division was similar to that observed on restoration of the full phosphate concentration

Table 1 Initiation of cell growth in deficient media

Additions	(1) Complete Labelled nuclei (%)	Missing nutrient		(3) Histidine+ glutamine		(4) Methionine		(5) Leucine	
		(2) Phosphate Labelled nuclei (%)	Cell no. ($\times 10^{-5}$)	Labelled nuclei (%)	Cell no. ($\times 10^{-5}$)	Labelled nuclei (%)	Cell no. ($\times 10^{-5}$)	Labelled nuclei (%)	Cell no. ($\times 10^{-5}$)
None	2	2	0.89	6.8	0.14	6	0.22	8	0.22
FGF	39	9	1.08	5.6	0.16	8	0.24	7	0.17
Insulin	10	52	1.57	53	0.26	28	0.27	10	0.19
FGF+I	60	55	1.64	62	0.24	29	0.28	18	0.25
Serum	77	75	1.40	80	0.31	82	0.41	62	0.34
Missing nutrient	2*	82	1.69	62	0.26	89	0.48	81	0.44

Swiss mouse 3T3-4A cells were plated in 2.5 ml of 10% dialysed foetal calf serum and Dulbecco's modified Eagle's medium (DEM) and grown for 12 d (5 d at confluency 0.94×10^5 cells per 3.5 cm dish). (1), As previously described (ref. 6). For the nutrient-depleted cultures 3×10^4 cells were plated into 10% dialysed foetal calf serum and DEM containing 1/100 of phosphate (2), 1/25 of histidine and glutamine (3), 1/25 of methionine (4) and 1/100 of the normal leucine (5) concentrations. After 3 d the cells reached final densities of 1×10^4 – 8×10^4 per dish. For phosphate and histidine plus glutamine-deprived cultures 93% to 94% of the cells stopped in the G_1 phase of the cell cycle, whereas cultures starved of methionine and leucine mainly ceased growth randomly¹⁰. Then 50 ng ml⁻¹ fibroblast growth factor (FGF), 250 ng ml⁻¹ insulin (50 ng ml⁻¹ in (1)), 20% dialysed foetal calf serum or the normal concentration of phosphate (2), histidine plus glutamine (3), methionine (4), leucine (5) were added. The initial concentration of phosphate in DEM was added to (1) (*). The fraction of cells with radioactively-labelled nuclei and the cell numbers per 3.5-cm dish were recorded as described previously⁶. For autoradiography the cultures were exposed to 3 μ Ci ml⁻¹ ³H-thymidine at 1 μ M from 8 to 36 h after additions throughout the entire DNA synthetic period in synchronised cultures. The percentage of cells with radioactively-labelled cell nuclei on addition of 0.5 μ g ml⁻¹ hydrocortisone (HC) was (1) HC alone 4%; FGF+HC, 40% (2) HC, 8%; FGF+HC, 11%; HC+I, 44%; FGF+HC+I, 60% (3) HC, 5%; FGF+HC, 10%; HC+I, 30%; FGF+HC+I, 70% (4) HC, 7%; FGF+HC, 8%; HC+I, 30% (5) HC, 8%; FGF+HC, 9%; HC+I, 20%. Serum was dialysed for 3 d against 10 volumes of isotonic saline with six changes at 4 °C. It then contained less than 1% of the amino acid concentrations present in the original preparations as determined in the amino acid analyser. In cultures where phosphate or histidine plus glutamine had been completely removed from the medium addition of insulin had no effect.

(Fig. 1). Similar results were obtained with quiescent 3T3-4A cells or human diploid fibroblasts¹⁵ exposed for 3 d or 5 d respectively to 1/100 of the normal glucose or phosphate concentrations. Addition of 250 ng ml⁻¹ of insulin doubled the number of cells by 40 h without any increase in the controls (not shown). In 3T3-4A cultures deprived of methionine and leucine, insulin induced only 30% and less than 10% increases in cell numbers and radioactively labelled nuclei respectively (Table 1). Addition of 20% dialysed foetal calf serum or the missing nutrient, initiated the majority (60–90%) of the cells to synthesise DNA and divide in all cultures.

The following control experiments (not shown) suggested that a contaminant¹⁶ was not responsible for insulin's growth-promoting effects. (1) Both 250 ng ml⁻¹ of EDTA-dialysed or non-dialysed insulin preparations initiated 30–50% of the phosphate or glutamine plus histidine-starved 3T3-4A cells to synthesise DNA and divide. (2) Extensively boiled insulin or insulin digested with papain bound to beads were completely unable to initiate DNA synthesis and cell division in similar conditions. In the case of other hormones, preparations of ovarian growth factor¹⁷ at 100 ng ml⁻¹ increased the incorporation of ³H-thymidine into DNA 10-fold and approximately doubled 3T3 cell numbers when added to quiescent cultures maintained in low phosphate (Table 2) or 1/25 of the normal glutamine (not shown) concentration. Hydrocortisone at 0.5 μ g ml⁻¹, oxytocin, vasopressin, glucagon and prolactin (not shown)—all at 50 ng ml⁻¹—had no effect, whereas prostaglandin E₁ at 30 μ g ml⁻¹ abolished the capacity of insulin to initiate cell division (Table 2).

Insulin may exert its growth-promoting effect by increasing the intracellular transport of the limiting nutrient^{7,8} since it predominantly increases uptake rates of the A (sodium-dependent) amino acid transport systems and not the L (sodium-independent) system¹⁸ in various tissues¹⁹. The A group includes glutamine, histidine, and methionine while the L group includes methionine and leucine¹⁹. Also EGF and insulin stimulate α -aminoisobutyrate uptake in cultured human fibroblasts²⁰. Direct changes in transport rates *per se* cannot solely explain the ability of insulin to initiate cell growth in cultures partially deprived of phosphate, since the same concentrations of FGF and insulin stimulate approximately the same increase in phosphate uptake in medium containing 1/100 of the normal phosphate ion concentration¹².

Here we have shown that insulin, unlike FGF, can initiate DNA synthesis and cell division in fibroblast cell cultures whose growth is completely dependent on the concentration of a number of limiting nutrients. Conversely, FGF initiates substantial increases in DNA synthesis and cell division in cultures allowed to become quiescent without artificial depletion of the culture medium, whereas insulin at the same low concentration has little effect. Thus the hormonal regulation of cell growth in cultures depleted of low molecular weight nutrients differs from that in cultures essentially deprived of serum. This suggests that growth-controlling polypeptide hormones may be divided into at least two distinct classes;

Table 2 Specificity of the initiation of cell growth in phosphate-deficient medium

Additions	³ H-DNA (c.p.m. $\times 10^{-5}$)	Cell no. ($\times 10^{-5}$)
None	0.25	0.69
Insulin	2.16	1.12
Oxytocin	0.17	0.82
Vasopressin	0.18	0.95
OGF	2.06	1.24
Hydrocortisone	0.22	0.45
Insulin + PGE ₁	0.32	0.64

Cells were grown as described in Table 1(2) and after 3 d in medium containing 1/100 of the normal phosphate concentration either 50 ng ml⁻¹ of insulin, oxytocin, vasopressin, 100 ng ml⁻¹ ovarian growth factor (OGF), 0.5 μ g ml⁻¹ hydrocortisone, or 30 μ g ml⁻¹ prostaglandin E₁ (PGE₁) were added as indicated. PGE₁ was added 10 min before insulin. The c.p.m. of ³H-thymidine incorporated into DNA and the number of cells were recorded as described in Fig. 1 except that the radioactive-labelling time was from 8 to 36 h after additions. Restoration of the normal concentration of phosphate yielded 1.36×10^5 cells.

those which depend for their action on general changes in certain intracellular nutrient concentrations and those which require additional intracellular signals for their mitogenic activity.

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A surface-active agent involved in PVC-induced haemolysis

MANY different forms of PVC powders are manufactured, the variety of the polymers depending on the initial constituents in the reaction mixture. Suspension homopolymers and paste (emulsion or microsuspension) polymers form 91% of the total PVC produced in the US. The latter products range in particle diameter from 0.5 to 30 μm and generally have ionic detergents added during their manufacture, whereas suspension homopolymers have no ionic

detergent and are of larger particle diameter (60-180 μm). A preliminary investigation¹ had shown that some forms of PVC particles produced haemolysis *in vitro* and it was suggested that this effect was due to some residual component of the preparation (not vinyl chloride monomer) located on the surface of the particle. The following data support these findings and suggest that the surface-active factor is ionic detergent, used in the manufacture of the paste polymers.

A range of PVC samples was tested for haemolytic activity; fourteen of these were obtained from normal production processes and were used without further treatment; eight samples were specially prepared so that the associated detergent and other factors were different in each case; and a further four samples (20-23) were normal production materials which had been subsequently treated with components (other additives) of the reaction mixture. The results shown in Table 1 are for representative samples of the different types of PVC preparation. Suspension homopolymers have no haemolytic activity unless experimentally treated with detergent (compare samples 19 and 3). A considerable mass of this type of polymer is necessary to produce a significant haemolytic action, and the detergent, its relative concentration and the presence of other additives are also critical factors (compare sample 19 and samples 9 and 8).

All the paste polymers of normal commercial origin or those experimentally produced showed some haemolytic activity although this was low when the detergent code C (a derivative of a sulphated oleate ester) was present (samples 12, 20, 21 and 23). Particularly strong haemolytic potential was found for PVC paste polymers containing code B detergent, sodium dodecyl benzene sulphonate (samples 10, 14 and 18) and in two samples of unknown composition. Paste polymers containing the detergent sodium lauryl sulphate (code A) produced significant haemolysis but were not as active as samples containing detergent B. The haemolytic activity of paste polymers with code A detergent was dependent on: the particle size of the PVC sample (samples 17 and 7 at 10 mg exposure); the total concentration of the detergents associated with the particle surface (samples 15 and 7); and the presence of additives and other detergents (samples 7, 20 and 22).

It has been reported previously¹ that PVC-induced haemolysis could be markedly reduced if the sample was first washed with water or saline and that once removed, the surface-active factor was too dilute or did not retain activity in solution. The present studies have, however, indicated

Table 1 Haemolytic potential of different PVC samples

PVC sample code number	Sample description	Particle size (μm)	Detergent code	% Haemolysis produced by			
				5 mg	10 mg	20 mg	50 mg
3	SH (NP)	60-120	Absent	—	1	—	1
8	SH (EP)	60-120	B	—	1	—	2
9	SH (EP) low detergent	60-120	A	—	1	—	3
19	SH (EP)	60-120	A†	5	7	13	83
5	Vinylchloride-Vinylacetate copolymer	60-180	Absent	—	0	—	0
7	Paste polymer (NP)	0.5-30	A†	10	99	—	90
15	Paste polymer (EP) low detergent	0.5-30	A†	20	38	100	94
17	Paste polymer (EP, slightly larger particle size distribution)	0.5-30	A†	13	33	99	96
12	Paste polymer (NP)	0.5-30	C	7	10	—	—
20	Paste polymer (as 12 plus other components)	0.5-30	C+A†	7	12	—	—
21	Paste polymer (as 12 plus other components)	0.5-30	C*	5	15	—	—
22	Paste polymer (as 12 plus other components)	0.5-30	C+A	8	81	—	—
23	Paste polymer (as 12 plus other components)	0.5-30	C*	4	7	—	—
10	Paste polymer (NP)	0.5-30	B	100	97	—	—
14	Paste polymer (EP)	0.5-30	B	99	100	—	—
18	Paste polymer (EP) plus sulphosuccinate	0.5-30	B	100	98	—	—
24	Paste polymer (NP)	Unknown	Unknown	99	99	—	—

Haemolysis experiments were carried out as described previously¹ in quadruplicate.

SH, Suspension homopolymer; NP, in normal production; EP, experimentally produced; A, sodium lauryl sulphate; B, sodium dodecyl benzene sulphonate; C, sulphated oleate ester-sodium or potassium; †, at least two additives present; *, single additive present. PVC sample codes are Chemical Industries Association identification numbers.

that some samples (10, 14, 18 and 24) still retain haemolytic potential after washing in buffered saline and that the first wash from 3 of these samples (10, 18 and 24) retains haemolytic potential (45–100%). It is possible that after centrifugation, some very fine particles of PVC remain in the wash material and so account for the haemolytic activity in the wash. Diameter size distribution data suggest that 30–50% of particles in some samples are below 2 μm . The results obtained by further processing the wash material through a 0.2- μm filter before testing for haemolytic potential would support this hypothesis. The wash from one sample (18) still produced 100% haemolysis after passage through the 0.2- μm filter although this effect was reduced to 4% by passage through a 0.01- μm filter. It could also be argued, however, that detergent in the washings binds to the Millipore filter thus reducing the haemolytic potential of the filtered washings.

Most of the paste polymers containing the detergent sodium dodecyl benzene sulphonate (10, 14 and 18) retained high haemolytic activity (75–100%) after washing in saline, an effect particularly pronounced with sample 18 which also contained sulphosuccinate. This effect was not found with PVC samples containing sodium lauryl sulphate (10–20%) which is more readily solubilised. The haemolytic potential of the former samples, however, was totally reduced if they were first treated with ethanol.

Thus, in summary, it is evident that suspension PVC homopolymers have little, if any, haemolytic potential, whereas paste or emulsion powders produce haemolysis probably due to the presence of ionic detergent on their surface. The extent of reaction of these latter PVC polymers is dependent on their particle diameter and size distribution, the concentration and type of detergent and the presence of other processing additives. The results also indicate that certain detergents such as sodium dodecyl benzene sulphonate form a tight association with PVC particles and in this form they are highly reactive membrane-lytic agents. The physiological implications of the above findings are as yet unclear and therefore need assessment by further experimentation.

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Pioneer neurones in an insect embryo

INSECT sense organs are produced by small groups of specialised epidermal cells¹. The receptor neurones differentiate at the surface, so developing sensory axons grow inwards from the epidermis to the central nervous system (CNS). How do they find their way? During larval life the axons of newly differentiated sense cells combine with those of neighbouring receptors and thus are guided to the nearest branch of a peripheral nerve which carries them to the CNS². At metamorphosis the axons of adult sensory neurones reach their central destination by growing along persistent larval nerves which are associated with the developing imaginal disks^{3–5}. Thus with pathways to the ganglia already established, growth along existing nerves ensures the delivery of each generation of sensory axons to within a few hundred micrometres of their central targets.

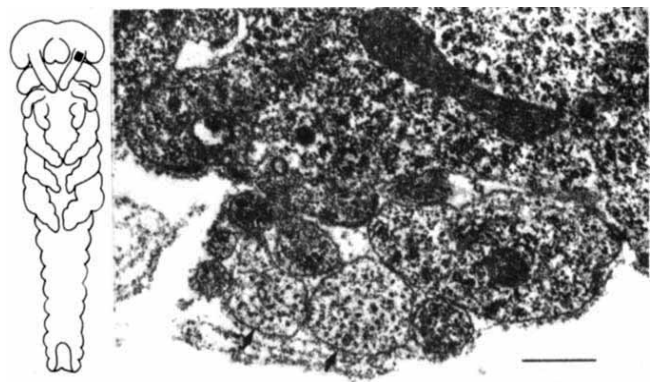


Fig. 1 One of two pairs of axons (arrowed) seen in transverse section through the base of the antenna at the level indicated in the inset. At 105 h of embryonic life at 30 °C. Scale: 1.25 μm .

Just how the connection between the surface and the CNS is first established, whether by an outgrowth of nerves from the centre or by pioneering axons which grow inwards from the surface has never been shown, although some descriptions of embryonic development imply that the first axons to enter the developing appendages are growing outwards from the CNS^{6,7}. Presumably these early centrifugal axons would provide a route for the later differentiating sensory fibres to follow in growth to the centre. Here, however, I report observations on the embryonic nervous system of *Locusta migratoria* which show that the first pathways between the epidermis and the central ganglia are formed by axons which grow inwards from peripheral neurones which differentiate early in embryonic life.

Before it rotates round the egg at blastokinesis⁸ the locust embryo consists of a segmented band of cells lying on a bed of yolk with its ventral side uppermost. The ganglia develop as a strip on either side of the midline and the base of each appendage is a focus which axons must pass on their way inwards or outwards from the CNS. Timed embryos (30 °C) from a laboratory culture of *Locusta* were processed for electron microscopy⁸ and transverse sections were cut through the base of the antennae and the limb buds to look for the early appearance of peripheral nerves.

An initial search revealed that from 110 h of embryonic life onwards, two pairs of axons appear at the base of the antenna (Fig. 1). Where do these axons come from and are they growing into or out of the CNS? Serial sections cut parallel to the long axis of the antenna show that each pair of nerves is produced by a pair of neurones at its distal tip (Fig. 2). From 95 h onwards the two cells of each pair put out processes which grow together to the base of the antenna and enter the embryonic deutocerebrum. Further series of sections in both planes confirm that there are no other axons in the antenna at this stage, so the two pairs of distal neurones initiate the development of the antennal nerve. During subsequent development the axons of differentiating receptors accumulate about the two sets of pioneering fibres to form two bundles of nerves in the lumen of the antenna. Although the first pairs of axons are naked, the developing bundles become enclosed in the sheathing process of glial cells which appear at intervals along the antenna.

Slightly earlier in development, single pairs of axons appear at the base of each of the three thoracic limb buds. As in the antenna, each pair of nerves is produced by a pair of peripheral neurones. In the limb bud the single pair of cells lies at the anterior margin of the distal end of the lumen (Fig. 2) and from 90 h onwards both of these neurones put out processes which grow inwards to the CNS. Fifteen hours later the two nerves with an expanded growth cone enter the embryonic neuropile from the base of

the limb bud (Fig. 3). Further series of sections confirm that these two axons are the first nerves to appear in each of the limb buds, so in the legs as in the antennae, the route from the epidermis to the CNS is pioneered by the ingrowing axons of twin peripheral neurones.

These early differentiating neurones are significant cells in the development of the nervous system. They form the first pathway from the surface to the centre, establish a framework for later innervation and contribute the first peripheral axons to the developing neuropile. During post-embryonic development ingrowing axons which fail to find a peripheral nerve trunk become lost between the epidermis and the basement membrane². The pioneering embryonic fibres reach the central ganglia independently of existing nerves. The axons of the antennal neurones grow directly to its base, but the axons of the limb bud neurones consistently follow a less direct route, which carries them from the anterior half of the distal end of the limb (Fig. 2) over the ventral surface of the leg to the posterior half of its base, where they enter the neuropile (Fig. 3). This indirect route suggests that the path to the CNS is determined not simply

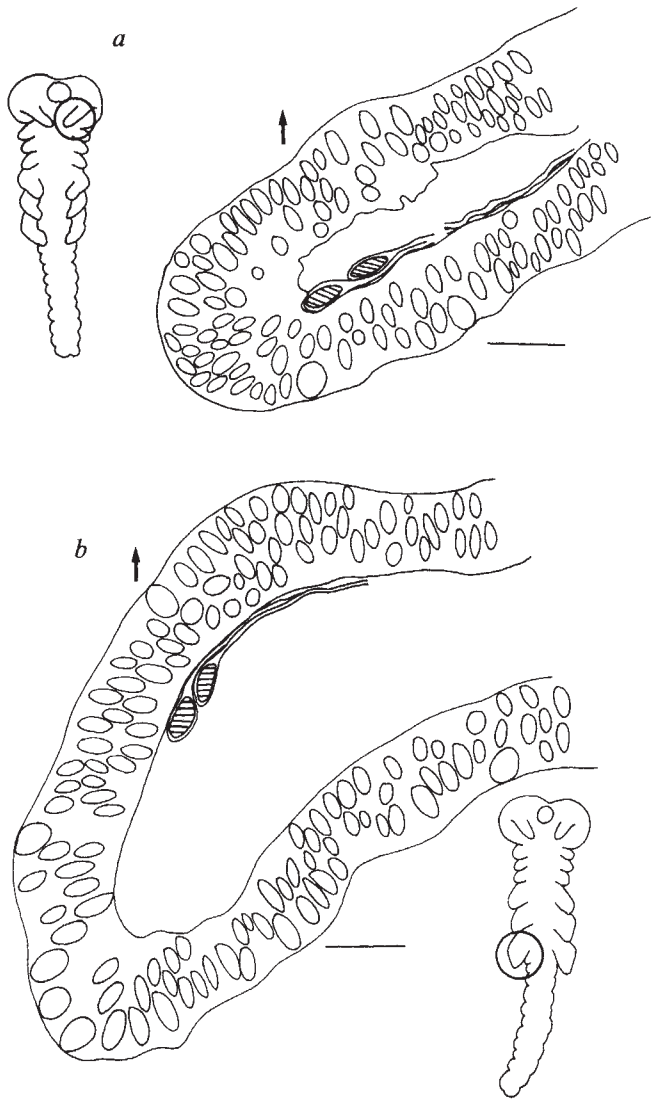


Fig. 2 *a*, One of two pairs of antennal neurones at 100 h of embryonic life. This pair lies on the ventral surface of the antennal lumen, the second pair on the opposite dorsal surface. *b*, Limb-bud neurones from the left metathoracic leg at 95 h of embryonic life. Both camera lucida drawings of 1-μm plastic sections. Arrows point anteriorly. Scales (both): 25 μm.

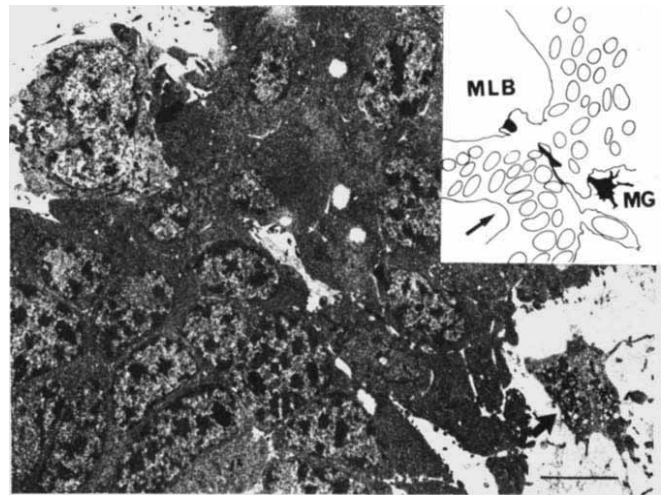


Fig. 3 Inset: camera lucida drawing of 1-μm section of growth cone entering embryonic metathoracic ganglion (MG) from the base of the metathoracic limb bud (MLB) at 105 h of embryonic life. Arrow points anteriorly. Electron micrograph shows part of the same area resectioned for electron microscopy. Notice large sheathing cell (arrowed top left) and expanded growth cone (arrowed bottom right) formed by the terminal expansion of the two axons. Scale: 4 μm.

by the orientation of the parent neurones but by an interaction between the growing axons and extrinsic cues in the limb bud. The sheathing cells are obvious candidates for a system of signposts. They occur consistently at intervals along the developing appendage and seem to provide a series of stepping stones between the tip of the limb and its base. The last of these cells occurs shortly before the point at which the axons are launched into the developing ganglion (Fig. 3).

At the stage at which the peripheral neurones first appear, differentiating central neurones are beginning to put out axons within the ganglia (C.M.B., unpublished) so the pioneer sensory fibres enter the CNS at a crucial stage in its development, when early differentiating ganglion cells lay the foundations of the future neuropile. Perhaps it is fortuitous that similar pairs of cells make the first peripheral pathways in both the antennae and the limb buds. Alternatively an equivalent ground plan may be established in different ganglia by fundamentally similar patterns of growth which reflect the segmental composition of the nervous system. In view of the influence of sensory innervation on the development of insect interneurones¹⁰ and the apparent inductive effect of ingrowing retinal axons on virgin ganglion cells in *Daphnia*¹¹ it is likely that the early appearance of sensory axons in the embryonic neuropile will be important in determining its structure as well as the distribution of later arriving sensory nerves. This too is now open to experiment.

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Influence of efferent retinal fibres on responsiveness of ganglion cells to light

EFFERENT retinal fibres originating in the nucleus isthmo-opticus^{1,2} of the pigeon have been shown, by electron microscopy, to terminate directly on amacrine cells³. These findings support the notion of a centrifugal control, originating at the brain, on the retina. Centrifugal fibres exist in several other non-mammalian species⁴, but their precise site of termination in the retina has not been determined. In the turtle, stimulation of centrifugal fibres at the level of the optic nerve has a powerful excitatory effect on amacrine cells since it produces a large, "all-or-none" excitatory postsynaptic potential (e.p.s.p.)⁵. Such excitation is in turn transmitted to ganglion cells⁵ through the extensive connections between amacrine and ganglion cells^{6,7}. Thus ganglion cells are influenced both by the visual input through photoreceptors and other retinal cells, and by a centrifugal input possibly mediated by amacrine cells alone. We describe here some preliminary results concerning the interactions between these two inputs to the ganglion cells.

We used eye-cups of turtles (*Pseudemys scripta elegans*) to which an optic nerve stump 1 cm long was left attached to stimulate both centrifugal fibres and ganglion cell axons. Because there are no reliable criteria to distinguish ganglion from amacrine cells based on their responses to light^{8,9},

ganglion cells were identified by recording intracellularly their antidromic response to stimulation of optic nerve fibres. A detailed description of the preparation and of the equipment used for intracellular recording is given elsewhere⁵.

In more than 50% of ganglion cells, from which intracellular recordings were obtained, illumination of their receptive field (600–800 μ m radius) produced photoresponses characterised by a transient depolarisation which did not show any appreciable centre-surround antagonism. These cells were selected for our experiment. Figure 1a shows the intracellular responses of a ganglion cell illuminated for 20 ms with a spot (left) and an annulus (right). It seems that the depolarising response maintains approximately the same time course irrespective of the area and pattern of illumination. The antidromic response of the same cell to stimulation of the optic nerve is shown in Fig. 1b. Here the spike response (marked by the arrow on the second line) to a single-shock stimulation of the optic nerve (0.5 ms, 2 mA) follows a direct spike produced by injecting outward current through the recording microelectrode. When the interval between the two spikes was reduced progressively, the antidromic response eventually disappeared because it collided with the direct spike travelling orthodromically along the same nerve fibre (Fig. 1b, third line). The collision interval found in this cell conforms with the values calculated from conduction times (about 12 ms) and refractoriness (about 8 ms).

When a train of stimuli is applied to the optic nerve, current intensities subthreshold for the antidromic response result in slow, graded, depolarising potentials. Graded responses of ganglion cells to stimulation of the optic nerve with a single shock are usually seen only with very high stimulus intensities (more than twice the threshold for the antidromic response)⁵. The greater efficacy of the low intensity, high frequency nerve stimulation to elicit synaptic

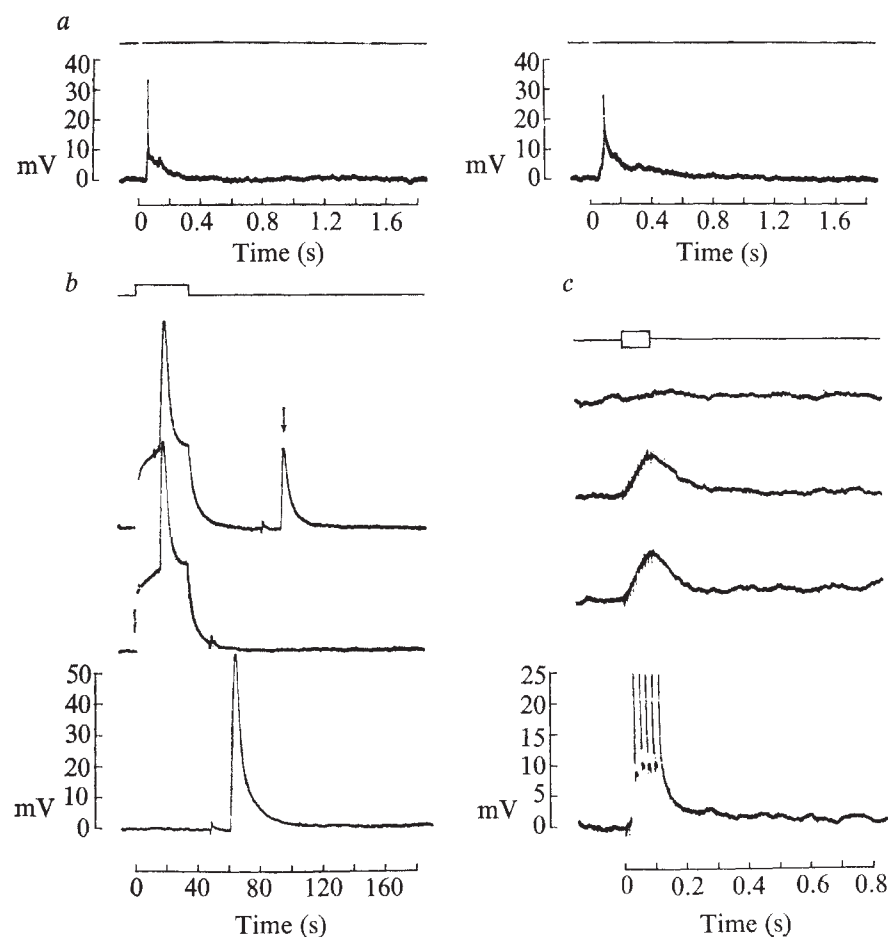
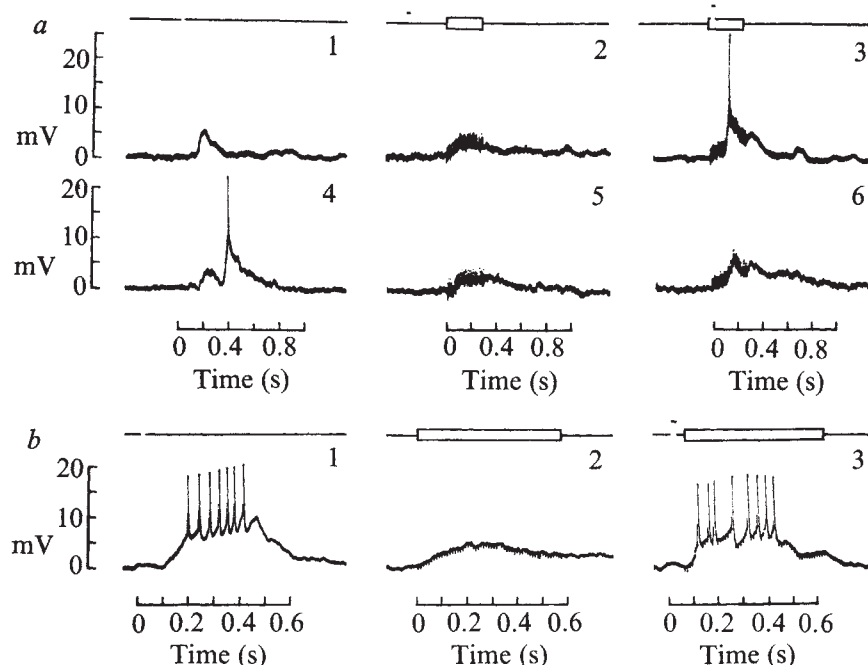


Fig. 1 Intracellular recordings from a ganglion cell in the turtle retina. *a*, Responses to illumination of a circle of 100 μ m radius (left) and of an annulus of 180 μ m and 1,000 μ m inner and outer radius, respectively (right). Zero time coincides with the flash indicated on the first line. *b*, Collision between the antidromic action potential (marked by the arrow on the second line) produced by optic nerve stimulation (0.5 ms, 2 mA) and the direct spike induced by artificially depolarising the cell membrane (third line). The injection of intracellular current is indicated on the first line. In the second line the antidromic action potential fails to invade the cell soma, but is fully developed when not preceded by the direct spike (fourth line). *c*, Ganglion cell responses to repetitive stimulation of the optic nerve (100 ms, 100 per s, 0.1 ms). The stimulus artefact is shown in the first line. The stimulus intensities used, for the responses from top to bottom, are 1.0, 1.5, 1.8 and 2.0 mA, respectively.

Fig. 2 Interaction between the ganglion cell responses to illumination and to stimulation of efferent fibres. *a*, Same cell as in Fig. 1. *a1*, Slow depolarisation produced by low intensity (4.8 log units of attenuation) illumination of a spot. The light stimulus is indicated in the first line. *a2*, Response to a train of stimuli (300 ms; 100 per s; 0.1 ms; 1.3 mA) applied to the optic nerve (the stimulus artefact is indicated in the first line). *a3*, Ganglion cell response to the combined visual and electrical stimulation. *a4*, Slow potential with a superposed spike produced by low intensity illumination of an annulus (3.6 log units of attenuation). *a5* and *a6*, as in *a2* and *a3*. *b*, Recordings obtained from a different ganglion cell. *b1*, Responses to a low intensity (3.6 log units of attenuation) illumination of a spot. *b2*, Response of the same cell to low intensity stimulation of the optic nerve (560 ms; 100 per s; 0.5 ms; 1.4 mA). *b3*, Ganglion cell response to the combined visual and electrical stimulation.



responses is probably due to a process of spatio-temporal summation by which progressively more low-threshold centrifugal fibres are recruited. If the strength of the repetitive stimulation was increased, the threshold for the antidromic response was eventually reached (Fig. 1c, fifth line). Thus, two distinct inputs to the same ganglion cell can be distinguished: one originating from photoreceptors excited by light (Fig. 1a), the other from activity in the centrifugal fibres (Fig. 1c).

The graded depolarisation produced by efferent fibres should affect the responsiveness of ganglion cells to a concomitant photic stimulation because of the resulting change in membrane excitability. Furthermore, since the influence of the centrifugal fibre on ganglion cells is mediated by amacrine⁸, ganglion photoresponses may be affected differently according to whether the visual input is conveyed from photoreceptors directly through bipolar cells, or through a more complex pathway involving amacrine cells. These two transmission mechanisms are likely to be activated when the photoresponses are elicited in central or peripheral regions of ganglion receptive fields, respectively^{9,10}.

Figure 2a1 shows a graded depolarising response to a 20-ms illumination of a 100- μ m radius spot centred on the recording electrode. High frequency, low intensity stimulation of the optic nerve produced the response shown in Fig. 2a2. By combining the two stimuli, the light-induced depolarisation seems to reach the threshold for an action potential (Fig. 2a3). On the contrary, a spike obtained by illumination of the periphery of the receptive field (Fig. 2a4) would not occur if a graded depolarisation were simultaneously produced by stimulation of the nerve (Fig. 2a6). On some occasions the response to the nerve stimulation may, at least partially, add to the annulus-graded photoresponse, but in these instances the centre photoresponse is always greatly enhanced. When centre illumination alone produces a repetitive spike discharge (Fig. 2b1), the centrifugal fibre stimulation may decrease the latency of the spike response (from about 200 to 100 ms) and slightly augment the discharge (Fig. 2b3). The centrifugal effects are mostly evident on photoresponses to low intensity illumination of either the centre or the periphery: when intensities 1.6–2.4 log units above threshold are used, the photoresponses are modified only slightly.

These results can be interpreted by assuming that a synaptic convergence at the level of the amacrine cells of the centrifugal input and the visual input from the periphery of the receptive field gives rise to an occlusive interaction, which prevents the two depolarising potentials from summing. But summation seems to be possible at the level of the ganglion cell, between the centrifugal input, mediated by amacrine cells and the visual input arising from the centre of the receptive field. This hypothesis may also explain the centrifugal effects on ganglion cell photoresponses observed in the chick¹¹.

It can be concluded that when a ganglion cell photoresponse coincides with the graded synaptic response to optic nerve stimulation an increased sensitivity to illumination of the central with respect to the peripheral areas is observed. This results in a redistribution of excitability over the ganglion cell receptive field. This effect may be of special functional significance for the detection of light stimuli of low intensity.

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Coupling between rod photoreceptors in a vertebrate retina

THE exquisite sensitivity of the scotopic visual system is well known to result, in large part, from a 'pooling' of the signals generated by the rod photoreceptors. It was once assumed that spatial integration of rod signals occurs at cellular sites proximal to the photoreceptors but recent

evidence indicates that, in turtle and toad retinæ, there is a marked spatial summation of the receptor potential recorded in a single rod¹⁻³. In the snapping turtle, *Chelydra serpentina*, we have estimated an individual rod to interact summatively with as many as 200 other rods³. Such pooling of signals at the input to the visual system will influence not only the sensitivity of the system but also spatial resolution of visual stimuli.

In the work reported here we demonstrate, by impaling pairs of cells, that the pathway of rod-rod interaction is not through the extracellular medium, nor is the horizontal cell an interneurone in the pathway. The interaction seems to be mediated by specific and direct pathways between rods that involve electrical and/or chemical synapses. The coupling in this pathway is summative and reciprocal. Furthermore, hyperpolarising currents are shown to be more effective than depolarising currents in eliciting responses in neighbouring rods.

We impaled and made simultaneous intracellular recordings from 27 pairs of neighbouring rods. We used an eyecup preparation of the snapping turtle retina³, into which two micropipette electrodes were advanced independently. This method is similar to that used by Baylor *et al.*⁴ on turtle cones. Rods were identified by their response characteristics, verified by intracellular injection of Procion yellow³. The separation between the rods of each pair was first determined by finding, for each rod, the positions at which brief flashes of a well focused light spot, 25 μm in diameter, elicited maximal responses. Extrinsic currents were then injected into each rod in turn, and were monitored directly by a current-sensing amplifier; the potential of the neighbouring rod was recorded simultaneously.

The results shown in Fig. 1 are from a typical experiment in which the rods were separated by 75 μm . In Fig. 1a the depolarising current (1.8 nA) evoked a depolarisation of about 2 mV in rod 2, whereas the hyperpolarising current (1.5 nA) evoked a hyperpolarisation of almost 9.5 mV. As a monitor of the cell integrity, a light stimulus was presented after each current injection. The slow hyperpolarisation on the right of each voltage record is the normal light response of the rod.

Essentially identical results were obtained when current was injected into rod 2 while monitoring the potential in rod 1. Figure 1b shows that depolarising and hyperpolarising current pulses of 1.8 nA, injected into rod 2, elicited in rod 1 a depolarisation of 1.2 mV and a hyperpolarisation of 8.3 mV, respectively.

The control records of Fig 1c were obtained when the electrode was withdrawn from rod 1 to a position nearby in the extracellular medium. They show the resulting intracellular potentials in rod 2 when current pulses of ± 1.6 nA were injected into the extracellular medium near rod 1. In all cases these potentials were less than 1 mV, much smaller than those recorded when both electrodes were intracellular. Similar results were obtained when the current-passing electrode was intracellular and the voltage-recording electrode was just outside the other cell. The small potentials that were still recorded in these conditions must have resulted from current flow in ground loops within the recording system, because they could be recorded even when the current-passing electrode was withdrawn completely from the eye.

Our results confirm that in the snapping turtle, the rod photoreceptors are functionally coupled to one another. More important, several additional points are demonstrated. First, the polarity of the coupled potential in one rod is the same as the potential induced by injecting current into the other rod. This is consistent with previous recordings from single rods, which indicate that the rod-rod interaction is summative. Second, the coupling between rods is reciprocal. Third, the magnitude of the coupled potential depends on the direction of extrinsic current; inward hyper-

polarising currents evoked larger coupled potentials than outward depolarising currents of the same strength. Fourth, since the effects of intracellularly injected currents could not be mimicked by injecting similar currents into the extracellular medium, we conclude that summative interactions between rods must be mediated by specific intercellular pathways.

Of the 27 pairs of rods from which recordings were made, 14 pairs were found to be coupled, and in all cases

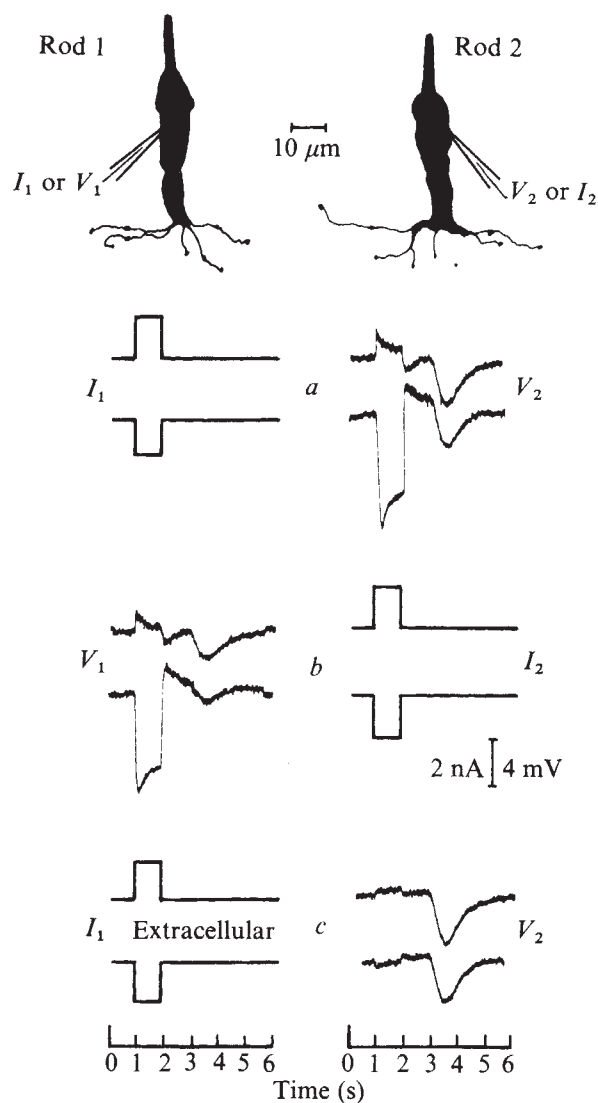


Fig. 1 Simultaneous intracellular recordings from two rods separated by 75 μm . Our recording situation is depicted schematically at the top, using camera lucida drawings of two snapping turtle rods that were delineated by injection of Procion yellow³. The current records, I_1 and I_2 , show the respective time courses of extrinsic current through rod 1 and rod 2. The potential records, V_2 and V_1 , show the respective responses of rod 2 and rod 1, first to a current pulse passed through the other impaled rod and then to a light flash. *a*, Depolarising (outward) current pulse of 1.8 nA and a hyperpolarising (inward) current pulse of 1.5 nA were passed into rod 1. The resulting potential changes are shown in the right column. The top records show the depolarising current in rod 1 and the associated potential change in rod 2; the hyperpolarising current and its effects are shown in the lower records. This convention is also followed in *b* and *c*. *b*, Two 1.8-nA current pulses of opposite direction were passed into rod 2. On the left are shown the potential deflections in rod 1. *c*, As a control, the electrode was withdrawn from rod 1 and current pulses of ± 1.6 nA were passed through its tip, while still recording in rod 2. The magnitude and direction of the potentials in all these recordings are given with respect to the resting membrane potential of the rod in darkness.

the coupling characteristics were as described. The maximum separation of coupled rods was about 110 μm . This compares with an upper limit of 150 μm which we found by determining the retinal area over which light responses summate in a single rod³. Of the 13 pairs of rods that were not coupled, the rods of 12 pairs were separated by more than 120 μm . For one pair, however, the separation was only 50 μm . In that case the injection of currents into either rod failed to evoke a potential in the other rod, although each rod was clearly influenced by the light responses of rods up to about 150 μm away. Baylor *et al.*⁴ showed that the cones of the red-eared turtle, *Pseudemys scripta elegans*, are summatively and reciprocally coupled over distances up to 50 μm . Rods of the snapping turtle exhibit a similar coupling over more than twice that distance.

The only interneurone known to be involved in receptor interactions is the horizontal cell, which has been shown by double impalement recordings to mediate antagonistic interaction between rather widely separated cones in the red-eared turtle⁴. It seems unlikely, therefore, that the horizontal cell would be involved in summative interactions between rods. In addition, the area over which rod signals are summative is much smaller than the receptive field of horizontal cells³. In this work we made many simultaneous recordings from a rod and a nearby L-type horizontal cell. When extrinsic currents up to ± 6 nA were injected into the horizontal cell, there was consistently no significant potential evoked in the rod. It thus seems likely that summative rod interaction is mediated by direct pathways between rods.

An interesting feature of snapping turtle rods is that numerous teledendria, up to 40 μm long, extend laterally across the retina from their broad synaptic terminals. These have been demonstrated by both Golgi staining (Leeper and Stell, personal communication) and Procion yellow injections³, and are depicted at the top of Fig. 1. The teledendria may constitute the pathways by which rods can interact selectively with each other.

The teledendria exhibit both *en passage* and terminal swellings not unlike the presynaptic structures found at chemical synapses in other parts of the nervous system⁵. In our work we could not detect the delay of a chemical synapse, because our electrode resistance of 300–400 M Ω resulted in a recording time constant of 10 ms. Thus the involvement of chemical synapses cannot yet be ruled out.

The greater effectiveness of hyperpolarising currents in evoking potentials in neighbouring rods may not result from the nature of the coupling itself, but from a non-linear current-voltage relationship in each rod. This is suggested by one experiment in which we impaled a single rod with two electrodes and noted that a hyperpolarising current produced a larger change of membrane potential than a depolarising current of equal strength. Thus further work must be done to elucidate the mechanism underlying the special efficiency of hyperpolarising responses in eliciting responses in neighbouring rods. Regardless of its basis, it seems likely that this effect is functionally significant, since the rod's normal light response is a hyperpolarisation.

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Diurnal rhythm in rat pineal cyclic nucleotide phosphodiesterase activity

MANY substances in the rat pineal organ exhibit marked diurnal variations; some of these fluctuations are circadian, that is, cued but not driven by environmental lighting. The content of the pineal hormone melatonin¹ for example, is seven- to tenfold higher during the dark phase of the diurnal cycle than during the light phase². Similarly, the activities of the melatonin-synthesising enzymes 5-hydroxytryptamine-*N*-acetyl transferase (NAT) and hydroxyindole-*O*-methyl transferase (HIOMT) are also higher in the dark (by factors of 15–70-fold and threefold, respectively^{3,4}). The immediate precursor of melatonin, *N*-acetyl 5-HT is 10–30-fold higher in the dark than in the light⁵. In contrast the pineal content of the precursor of *N*-acetyl 5-HT, 5-HT is twofold higher in the light than in the dark phase of the diurnal cycle⁶, suggesting that changes in NAT activity regulate changes in melatonin biosynthesis. These changes have been shown to be controlled by environmental light acting through the retina by way of central nervous pathways to the superior cervical ganglion, and thence through postganglionic sympathetic fibres to the pineal body⁷. Noradrenaline released from these fibres acts on β adrenoreceptors on the parenchymal pineal cells to stimulate adenylate cyclase, causing an increase in intracellular cyclic AMP which is followed by increased NAT activity and melatonin biosynthesis^{8,9}. Decentralisation, superior cervical ganglionectomy, administration of β -adrenoreceptor blocking drugs or protein synthesis inhibitors can all block the light-dark changes observed in NAT activity in this organ^{10,11}.

We have recently shown that stimulation of adenylate cyclase by cholera toxin is also an effective stimulus for the induction of NAT in rat pineal organ cultures. The increases in adenylate cyclase activity caused by cholera toxin also lead to increases in the cyclic AMP hydrolysing enzyme, cyclic nucleotide phosphodiesterase (PDE), so that the increases in cellular cyclic AMP content caused by cholera toxin are only transient¹².

In view of the importance of PDE in modifying intracellular cyclic AMP levels, and thereby cellular responsiveness to β -adrenoreceptor stimulation, we have studied the physiological regulation of this enzyme in a diurnal lighting situation. We report here that PDE activity in the rat

Table 1 Effect of superior cervical ganglionectomy and (–)-propranolol on dark-induced increase in pineal PDE activity

Treatment	Time of death	Cyclic AMP hydrolysis	Cyclic GMP hydrolysis
Sham	1200 light	0.9 $\pm$ 0.15	1.9 $\pm$ 0.24
Ganglionectomy	1200 light	1.0 $\pm$ 0.13	1.9 $\pm$ 0.31
Sham	2400 dark	1.6 $\pm$ 0.11*	2.9 $\pm$ 0.25*
Ganglionectomy	2400 dark	1.0 $\pm$ 0.22	2.1 $\pm$ 0.43
Saline	1200 light	0.87 $\pm$ 0.09	1.7 $\pm$ 0.24
(–)-Propranolol	1200 light	0.84 $\pm$ 0.05	1.6 $\pm$ 0.12
Saline	2400 dark	1.50 $\pm$ 0.13*	2.7 $\pm$ 0.22*
(–)-Propranolol	2400 dark	0.93 $\pm$ 0.10	1.8 $\pm$ 0.17

Rats were ganglionectomised bilaterally under Equithesin anaesthesia, or sham operated; allowed to recover for 1 week, and killed at noon and midnight of a 12 h light–12 h dark schedule. Pineals were quickly removed and frozen and PDE activity was determined (see legend to Fig. 1). Other animals were injected with (–)-propranolol (20 mg kg^{–1}) or 0.9% saline subcutaneously at 2000 or 0800 and killed at 2400 and 1200 respectively, the pineals removed and PDE determined. Each point is the mean of 5 pineals $\pm$ s.e.m. Values are expressed as nmol hydrolysed per 10 min per pineal. **P* < 0.001 as compared with noon control values.

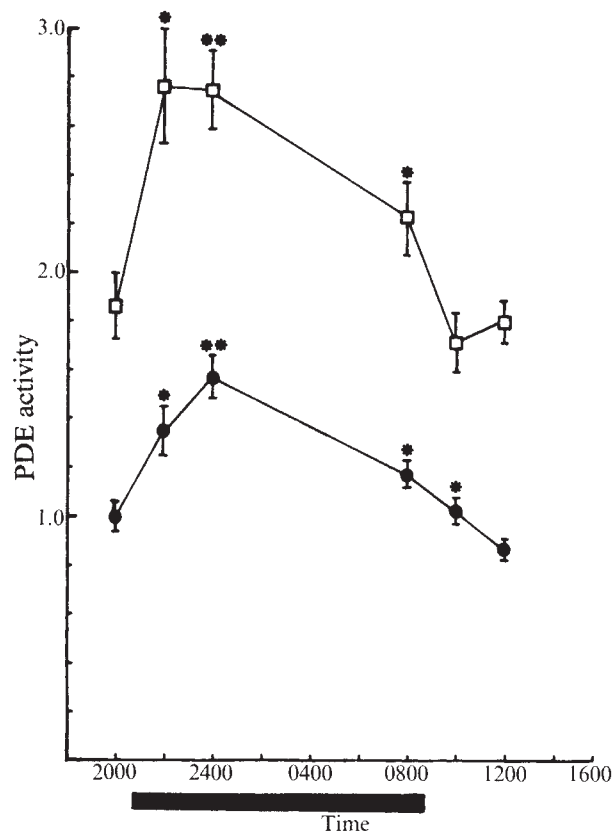


Fig. 1 Diurnal variation in cyclic nucleotide phosphodiesterase in rat pineal organ. Rats were maintained on a 12 h light–12 h dark schedule, with lights on from 0900 to 2100 and were killed at times indicated, and pineals removed and frozen. A single pineal was homogenised in distilled water (100 μ l) and PDE activity determined in an aliquot by the method of Thompson and Appleman¹³. Values were corrected for recovery losses of adenosine and guanosine according to the method of Lynch and Cheung¹⁴. ●, Cyclic AMP hydrolysis at 10^{-6} M substrate concentration; □, cyclic GMP hydrolysis at 10^{-6} M substrate concentration. Each point is the mean of 5 animals. Vertical bars indicate $\pm$ s.e.m. Values are expressed as nmol hydrolysed per 10 min per pineal. ** $P < 0.001$; * $P < 0.05$ compared with noon values.

pineal is significantly higher during the dark phase of a diurnal lighting regimen, and that this diurnal rhythm seems to be associated with increased β -adrenoceptor stimulation during the dark phase.

Male Sprague–Dawley rats (150–200 g) were housed in groups of 10 in a controlled lighting situation, with lights on from 0900 to 2100; the animals were given food and water *ad libitum*. Light was provided by a 60-W incandescent bulb 60 cm from the cage, and temperature was constant at 23–24 °C. In some animals, bilateral superior cervical ganglionectomy was performed under Equithesin anaesthesia. Rats were decapitated at various times during

the light or dark periods, the heads were chilled on ice and the pineal body quickly removed and stored at -20 °C before assay. Cyclic nucleotide phosphodiesterase (PDE) activity was determined by the method of Thompson and Appleman¹³ and values were corrected by the method of Lynch and Cheung¹⁴ (see legend to Fig. 1).

PDE activity in the rat pineal organ exhibited a 24-h rhythm for the hydrolysis of both cyclic AMP and cyclic GMP (Fig. 1), with the highest values occurring in each case in the dark. At 1μ M substrate concentration maximal cyclic AMP hydrolysis in the dark at 2400 was 183% of that at 1200 in the light (Fig. 1), and cyclic GMP hydrolysis at 2400 was 162% of that measured in the light (Fig. 1). Since both cyclic AMP and cyclic GMP are hydrolysed with complex kinetic behaviour in the pineal body (K.P.M. and L.L.I., in preparation), phosphodiesterase activity was also measured at higher concentrations of each nucleotide (100 μ M). Both cyclic AMP and cyclic GMP hydrolysis were again maximally increased by 200% in the dark compared to the light values at this substrate concentration.

To determine whether the diurnal rhythm in pineal cyclic nucleotide PDE was controlled by changes in sympathetic nerve activity in the postganglionic fibres innervating the pineal organ, bilateral superior cervical ganglionectomy was performed in some animals. These were killed a week after the operation at midnight and at noon, and pineal PDE measured (Table 1). Ganglionectomy completely blocked the dark-induced increase in pineal PDE, ganglionectomised animals having PDE levels not significantly different from sham-operated controls killed in the light. This suggests that increased sympathetic activity in the dark is responsible for the increased pineal PDE. To determine whether the changes in PDE activity were mediated by noradrenaline release, the β -adrenoceptor blocking agent (–)-propranolol was injected 1 h before the lights went off (2000), or 1 h before the lights went on (0800). PDE activity was again determined at noon and midnight (Table 1). (–)-Propranolol blocked the dark-induced increase in pineal PDE activity, propranolol-treated animals having PDE levels not significantly different from saline-injected controls killed in the light.

Treatment with the protein synthesis inhibitor cycloheximide 1 h before the lights went off (2000), or 1 h before the lights went on (0800) partially suppressed the dark-induced increase in pineal PDE (Table 2). At midnight PDE levels in the cycloheximide-treated animals were still significantly increased over noon control values, being 125% of the latter for both cyclic AMP and cyclic GMP hydrolysis.

These results are consistent with the recent findings that administration of the β -adrenoceptor agonist isoprenaline *in vivo* can increase PDE activity in the rat pineal organ¹⁶. The diurnal changes observed in PDE activity in the pineal may result from an increased synthesis of PDE enzyme protein in the dark, or possibly through changes in the content of an endogenous activator. In the rat adrenal medulla, an increased concentration of an endogenous activator for PDE has been shown to occur within 20 min after carbamylcholine injection *in vivo*¹⁵, although there is as yet no evidence for the occurrence of such changes in the pineal organ.

Since changes in cyclic AMP seem to be sufficient for the induction of NAT activity in the pineal body¹², and since increases in PDE activity lead to decreases in cyclic AMP content¹², it is clear that alterations in PDE activity can lead to alterations in the sensitivity of pineal biosynthetic enzymes to β -adrenoceptor stimulation, by altering the half life of the intracellular mediator cyclic AMP. The fact that twofold changes in the activity of this enzyme occur in normal physiological conditions is interesting, since diurnal changes in the sensitivity of pineal biosynthetic enzymes to β -adrenoceptor stimulation also occur normally in controlled lighting regimens. Receptor sensitivity

Table 2 Effect of cycloheximide on dark-induced increase in pineal PDE activity

Treatment	Time of death	Cyclic AMP hydrolysis	Cyclic GMP hydrolysis
Saline	1200 light	0.72 ± 0.05	1.81 ± 0.14
Cycloheximide	1200 light	0.57 ± 0.10	1.6 ± 0.35
Saline	2400 dark	$1.25 \pm 0.1^{**}$	$2.6 \pm 0.13^{**}$
Cycloheximide	2400 dark	$0.89 \pm 0.04^{*}$	$2.3 \pm 0.06^{*}$

Rats were injected at 2000 and 0800 with 30 mg kg^{-1} cycloheximide or 0.9% saline subcutaneously, and killed 4 h later at 2400 and 1200 respectively; pineals removed, and PDE activity determined. Each point is the mean of 5 animals $\pm$ s.e.m. Values are expressed as nmol hydrolysed per 10 min per pineal. ** $P < 0.001$ * $P < 0.05$ compared with saline-treated controls killed at noon.

is highest during the light period, when PDE activity is lowest, and receptor sensitivity is lowest during the dark period, when PDE activity is increased¹⁷. Since it seems probable that PDE is induced by high levels of its substrate, cyclic AMP¹², its activity will, therefore, be high after periods of intense receptor stimulation and low after periods of receptor inactivity. The phenomena of sub- and supersensitivity could thus be at least partially explained by a shift in the dose-response curve of adenylate cyclase activation on intracellular cyclic AMP content, controlled by alterations in the rate of degradation of intracellular cyclic AMP. Alterations in ³H-alprenolol binding have recently been observed in sub- and supersensitive pineals¹⁸, suggesting that changes in the population of available receptors may also occur; such changes have also been shown to occur with a marked diurnal cycle¹⁹. Thus it is probable that several mechanisms contribute to changes in adrenoceptor sensitivity and therefore regulate tissue responses to catecholamine stimulation. For example, superior cervical ganglionectomy causes a striking supersensitivity in the accumulation of cyclic AMP and induction of NAT on β -adrenergic stimulation in daytime rats²⁰, as well as an increased adenylate cyclase response^{21,8}; and our results demonstrate that these phenomena cannot be explained in terms of changes in PDE activity, as the PDE levels are not changed in ganglionectomised animals.

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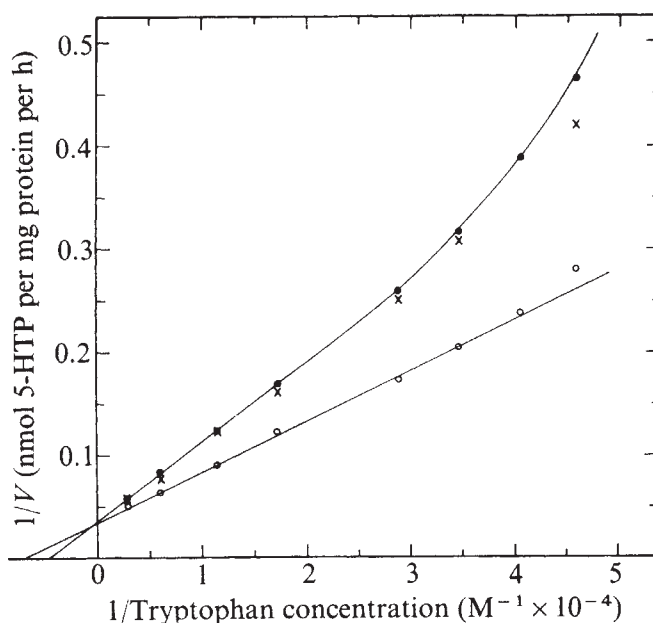


Fig. 1 Double reciprocal plots of tryptophan hydroxylase activity against tryptophan concentration. Effects of *in vivo* methiothepin (○) or methiothepin-LSD treatment (×); ●, control. Methiothepin and LSD were injected as described in Table 2. Soluble enzyme was prepared from the brainstem of rats. Tissues were homogenised (20 s) with a Polytron PT10,OD apparatus in 3 volumes 0.05 M Tris acetate, pH 7.6, containing 1 mM β -mercaptoethanol. Homogenates were centrifuged at 30,000g for 30 min at 4 °C and the supernatant was used as the final enzyme solution. Tryptophan hydroxylase activity was assayed in the presence of 0.32 mM 6-MPH₄ and various concentrations of tryptophan as described previously¹² except that the incubation period was shortened to 15 min. In these conditions, the formation of 5-hydroxytryptophan (5-HTP) was linear with respect to both time of incubation and protein concentration (0.5–1.2 mg ml⁻¹) in the assay mixture. The reaction was stopped by addition of 0.2 ml 4 N perchloric acid. Precipitated proteins removed by centrifugation were estimated by the procedure of Lowry *et al.*¹³ using bovine serum albumin as standard. 5-HTP was determined in aliquots (0.5 ml) of supernatant by the OPT spectrofluorimetric method¹² or by its native fluorescence in 3 N HCl (ref. 1) or 4 N HClO₄. Blanks were made by adding 6-MPH₄ at the end of the incubation. For the calculations, standards were made by adding known amounts of 5-HTP (1–2 μ g) to some samples just before incubation. Statistical calculations were done according to Snedecor and Cochran¹⁴. Apparent K_m and V_{max} were determined by double reciprocal plotting. Each point is the mean of three determinations carried out with tryptophan hydroxylase extracted from groups of 8 rats.

possibly involved, rapid changes in the enzyme affinity for its substrate and/or its cofactor may take place. Indeed, such changes are responsible for the activation of tyrosine hydroxylase seen in dopaminergic terminals after blockade of dopaminergic receptors by neuroleptics⁹. We used methiothepin(1-[10,11-dihydro-8-(methylthio)-dibenzo(b,f)thiepin-10-yl]-4-methylpiperazine maleate), a potent blocker of central serotonergic receptors¹⁰, to look for a similar activation of tryptophan hydroxylase. Our results indicate that the affinity of this enzyme for its substrate is markedly enhanced in methiothepin-treated tissue.

In preliminary experiments, male Sprague-Dawley rats (250–300 g) kept in a controlled environment for 10 d, were injected intraperitoneally with methiothepin (20 mg kg⁻¹) and killed 90 min later by decapitation. Slices of the brainstem were prepared and incubated for 30 min at 37 °C in the presence of ³H-tryptophan. In these conditions (described in detail elsewhere¹¹) the rates of formation of ³H-5-HT and ³H-5-hydroxyindole acetic acid (³H-5-HIAA) were markedly stimulated when compared with those in control tissues (+61%). This effect was related partly to an enhanced accumulation of ³H-tryptophan in slices (+30%); however, it was also due to an activation of the rate of tryptophan hydroxylation, as the conversion index

***In vivo* and *in vitro* activation of soluble tryptophan hydroxylase from rat brainstem**

TRYPTOPHAN hydroxylation is the rate-limiting step in the biosynthesis of 5-hydroxytryptamine (5-HT) in central serotonergic neurones. In physiological states, tryptophan hydroxylase (EC 1.14.16.4), the key enzyme, is not saturated by its substrate¹. Thus, three factors which modulate the concentration of tryptophan in brain—the levels of free tryptophan in plasma^{2,3}, the ratio of total tryptophan levels to those of other neutral amino acids⁴, and the uptake process for tryptophan in brain tissues^{5,6}—are important in the control of 5-HT synthesis. On the basis of indirect evidence^{7,8}, it was suggested that tryptophan hydroxylase could also play a regulatory role. Among the mechanisms

Table 1 Effects of various pharmacological treatments on soluble tryptophan hydroxylase activity from rat brainstem

Treatment	Tryptophan hydroxylase activity
Saline	3.14 ± 0.18
Methiothepin	4.21 ± 0.22*
LSD	3.20 ± 0.12
LSD-methiothepin	2.98 ± 0.24
Apomorphine	3.49 ± 0.15
Apomorphine-methiothepin	4.54 ± 0.28†

Methiothepin (20 mg kg⁻¹ intraperitoneally) was injected 140 min before death. LSD (1 mg kg⁻¹ intraperitoneally) was injected twice, 160 and 120 min before death. The same schedule was used for apomorphine administration (each 5 mg kg⁻¹ intraperitoneally). Tryptophan hydroxylase was assayed as described previously¹² with 0.16 mM 6-MPH₄ and 0.24 mM tryptophan. The enzymatic activity is expressed in nmol 5-HTP formed per mg protein and per h. Each value is the mean ± s.e.m. of determinations made on groups of 8 rats.

**P* < 0.05 compared with saline-treated rats.

†*P* < 0.05 compared with saline- or apomorphine-treated rats.

of tryptophan into 5-hydroxyindoles (³H-5-hydroxyindoles/tryptophan specific activity) was 33% higher in slices of treated rats. A similar, but less pronounced stimulatory effect (+24%) of methiothepin on *in vitro* 5-HT synthesis was observed when the drug (30 μM) was added directly to the incubating medium. This could be prevented when slices were incubated in the presence of both methiothepin (30 μM) and LSD (10 μM, D-lysergic acid diethylamide bitartrate, Sandoz) a well known agonist of 5-HT receptors.

When estimated in the presence of subsaturating concentrations of tryptophan (0.24 mM) and cofactor (0.16 mM) the activity of tryptophan hydroxylase isolated from tissues of methiothepin-pretreated rats (20 mg kg⁻¹ intraperitoneally) was increased by 30–40% (Table 1). This stimulation was observed from 90 min to 5 h after administration of the 5-HT blocking agent, whereas methiothepin incubated directly with the control enzyme was ineffective and inhibited the enzyme activity at high concentrations (–27% at 50 μM). LSD injected twice (each 1 mg kg⁻¹ intraperitoneally) 20 min before and 20 min after methiothepin (20 mg kg⁻¹ intraperitoneally) prevented the stimulation of tryptophan hydroxylase estimated 140 min after the 5-HT antagonist injection (Table 1). The potent agonist of dopaminergic receptors, apomorphine (5 mg kg⁻¹ intraperitoneally, Hoffmann-La Roche) injected 20 min before and 20 min after methiothepin did not alter the activation of tryptophan hydroxylase induced by the 5-HT blocking agent (Table 1). In the presence of a high concentration of tryptophan (0.5 mM), control tryptophan hydroxylase exhibited an apparent *K_m* for

the pteridine cofactor, 2-amino-4-hydroxy-6-methyl-5,6,7,8-tetrahydropteridine. (6-MPH₄ Calbiochem), equal to 183 μM. The enzyme isolated from the brainstem of methiothepin-treated rats exhibited the same affinity for the cofactor and its *V_{max}* was slightly but not significantly increased (Table 2). Conversely, methiothepin treatment increased significantly the affinity of the enzyme for tryptophan (Table 2) without changing its *V_{max}*. The determination of these kinetic parameters was carried out with a subsaturating concentration of 6-MPH₄ (0.32 mM) as the reduced pteridinic cofactor inhibited tryptophan hydroxylase activity when its concentration reached 0.4 mM (M.H. and S.B., unpublished). After double reciprocal plotting, the curve obtained with the enzyme extracted from control tissues was parabolic with an upward concavity suggesting a cooperative effect (Fig. 1), (Hill plot gave an *n* value close to 2). In contrast, the curve became linear for the enzyme of methiothepin-treated rats. As shown in Fig. 1, when rats were treated as in the previous experiment both with methiothepin and LSD, the affinity of the enzyme for tryptophan returned to the control value.

The change in affinity of the enzyme for its substrate in methiothepin-treated rats as well as the parabolic curve observed for the enzyme of control tissues (Fig. 1) suggest that tryptophan hydroxylase shares allosteric properties with other polymeric enzymes. Such enzymes can be activated by mild trypsinisation or exposure to small concentrations of detergents. Therefore, the effects of trypsin (1 : 250, Difco) and sodium dodecyl sulphate (SDS, Sigma) on the kinetic characteristics of the enzyme were examined. A tryptophan hydroxylase solution was incubated at 30 °C for 30 min in the presence of trypsin (6.5 μg proteolytic enzyme per mg protein in the brainstem supernatant). The reaction was stopped with 500 μg soybean inhibitor (Worthington) and the enzyme activity assayed as in Fig. 1. In these conditions, the affinity of tryptophan hydroxylase for tryptophan was markedly increased (Table 2). The *V_{max}* was reduced but a similar change occurred when the enzyme was preincubated for 30 min at 30 °C in the absence of trypsin, indicating that this alteration was simply related to the instability of tryptophan hydroxylase. When the enzyme assay was carried out in the presence of 0.01% detergent, a significant change in the enzyme affinity for its substrate was also seen. Both with trypsin and SDS, the affinity of tryptophan hydroxylase for the cofactor as well as its *V_{max}* (in the presence of 0.5 mM tryptophan) were significantly increased (Table 2).

The changes in 5-HT synthesis induced by the 5-HT antagonist, methiothepin, may be related to an increased transport of tryptophan in tissues, but also to an activation of tryptophan hydroxylase. At it could be directly observed *in vitro*, the affinity of the enzyme for its substrate amino acid was selectively

Table 2 Effects of various treatments on kinetic parameters of soluble tryptophan hydroxylase from rat brainstem

	Tryptophan		6-MPH ₄	
	<i>K_m</i> (μM)	<i>V_{max}</i>	<i>K_m</i> (μM)	<i>V_{max}</i>
Control	234 ± 17 (11)	22.1 ± 1.4	183 ± 15 (8)	23.7 ± 1.6
Methiothepin	139 ± 16* (5)	23.1 ± 2.4	181 ± 17 (5)	26.1 ± 0.8
SDS	110 ± 5* (5)	27.0 ± 3.0	102 ± 11* (5)	46.4 ± 5.2*
Preincubated control	213 ± 22 (6)	11.2 ± 0.8	166 ± 14 (5)	12.2 ± 0.9
Trypsin	106 ± 6* (6)	12.3 ± 1.1	104 ± 12* (4)	19.1 ± 1.5*

Determinations of apparent *K_m* and *V_{max}* of tryptophan hydroxylase with tryptophan concentrations in the range 20 μM–0.5 mM were carried out in the presence of 0.32 mM 6-MPH₄. When the concentration of 6-MPH₄ was the variable, tryptophan concentration was maintained at 0.5 mM. *In vivo* treatment with methiothepin (20 mg kg⁻¹ intraperitoneally) occurred 120 min before death. SDS (0.01%) was added to the standard assay mixture without preincubation. The effect of trypsin was analysed after 30-min preincubation of tryptophan hydroxylase solution with the proteolytic enzyme (6.5 μg trypsin per mg protein of the brainstem supernatant) at 30 °C. 'Preincubated control' corresponds to tryptophan hydroxylase solution incubated for 30 min at 30 °C in the absence of trypsin. *V_{max}* is expressed in nmol 5-HTP formed per mg protein and per h. Each value is the mean ± s.e.m. of determinations made in 4–11 experiments (numbers of experiments indicated in parentheses).

**P* < 0.05 compared with respective control values.

increased by methiothepin treatment. This is in contrast to the effect of methiothepin on tyrosine hydroxylase: indeed, this drug, which also blocks dopaminergic receptors, increased the affinity of the latter enzyme for its cofactor but not for its substrate⁸. As in the case of apomorphine, which antagonises the activation of tyrosine hydroxylase induced by neuroleptics⁹, LSD was able to prevent the increase in affinity of tryptophan hydroxylase for tryptophan after methiothepin administration. The activity of tyrosine hydroxylase is stimulated by incubating the soluble enzyme in the presence of small concentrations of trypsin¹⁵ or detergents¹⁶. A similar phenomenon was observed in our experiments with tryptophan hydroxylase. This activation was related to increased affinities for both the cofactor and the substrate. As already proposed for tyrosine hydroxylase, it may be postulated that tryptophan hydroxylase exhibits allosteric properties which could have a major role in the regulation of 5-HT synthesis.

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Purification of inactive phenylalanine hydroxylase protein from liver in classical phenylketonuria

THE molecular defect in classical phenylketonuria (PKU) has not been elucidated, but it seems certain that the block in metabolism is in the conversion of phenylalanine to tyrosine¹ and that the enzyme which carries out this reaction, phenylalanine hydroxylase (EC, 1.14.3.1), is defective². Progress in delineating the molecular defect has been slow because of the limited tissue distribution of phenylalanine hydroxylase, lability of this enzyme after death³, scarcity of normal and PKU livers for study and the nonspecific purification methods for this enzyme. We recently developed a specific, one-step method for purification of phenylalanine hydroxylase from crude extracts of monkey liver⁴, and have now applied it to crude extracts of livers from two human foetuses, from three patients who died of unrelated disease and from a patient with classical PKU who died at 12 yr. (The PKU patient did not follow the rapidly progressive

course described recently in variant forms of PKU⁵ and dihydropteridine reductase activity was demonstrated in the liver.)

This procedure yielded from PKU liver a protein which appears identical in molecular weight to the smaller of the

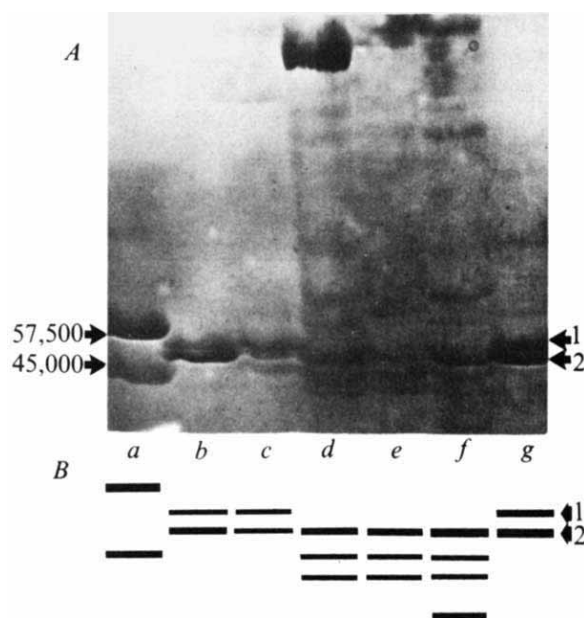


Fig. 1 Photograph (A) and the drawing (B) of analysis by SDS-Gradiopore (gel 2.5% to 27% acrylamide) of preparations obtained from crude extracts of various livers by affinity chromatography on a reduced pteridine affinity adsorbent (2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine-CH-Sepharose). The crude extract was passed through the column at a high salt concentration (0.4 or 0.8 M NaCl). After reducing the salt concentration (to 0.2 M), proteins were eluted with phenylalanine (0.1 M). Modifications to the published procedure⁴ involved the following. (1) For the foetal livers, the NaCl concentration was 0.4 M instead of 0.8 M in the extract and the high salt buffer; (2) for autopsy livers, sonication was used to release particulate enzyme⁸ and dialysis was used to prevent interference with binding to the resin by phenylalanine present in the tissue (especially necessary with PKU liver); (3) for all human liver EDTA was omitted from all column buffers. The Gradiopore gel was pre-equilibrated with reservoir buffer (2.4 g Tris base, 11.5 g glycine, 1 g SDS per l distilled water, pH 8.7) for 1.5 h at 200 V, and samples were applied in 2% SDS, 5% mercaptoethanol and 20% glycerol after heating at 100 °C for 1.5 min. Samples were then run for 17 h at room temperature at 75 V. The gel was stained with Coomassie brilliant blue R 250 (0.25% in 20% trichloroacetic acid and 20% methanol) overnight at 37 °C and destained overnight at 37 °C in 5% acetic acid using wool destaining pads. Samples illustrated are (a) 2.5 µg catalase plus 2.5 µg ovalbumin; (b) 3.7 µg monkey liver preparation (specific activity 0.43 U per mg protein); (c) 3.2 µg human foetal liver preparation (16 weeks gestation, normal) specific activity 0.37 U per mg protein); (d) 9.2 µg autopsy PKU liver preparation (15-yr-old, died of pneumonia) (specific activity 0); (e) 7.6 µg autopsy human liver preparation (4-yr-old spina bifida, hydrocephalic) (specific activity trace); (f) 11.2 µg human foetal liver enzyme (34 weeks gestation, hydrocephalic) (specific activity 0.01 U per mg protein); (g) 4.8 µg monkey liver enzyme (prepared with a modification of the published⁴ procedure whereby a precolumn of 2 ml of CH-Sepharose was used) (specific activity 0.5 U per mg protein). One unit of phenylalanine hydroxylase forms of 1 µmol of tyrosine per min at 37 °C. Only the part of the gel that contained bands was photographed. It represents an approximate range of acrylamide concentration of 13% (top of figure) to 26%. Phenylalanine hydroxylase proteins 1 and 2 (right of figure) and the molecular weights (left of figure) of the markers (catalase 57,500 and ovalbumin 45,000) are indicated. When a run was made only until the marker dye reached the end of the gel (3 h) an extra two strongly stained bands were seen in samples d-f; they were weakly stained in samples b, c and g. The molecular weights were about 12,000 and 10,000. The large molecular weight material (approximately 300,000) which is particularly strong in the PKU sample may be undissociated complex. The relevant bands are illustrated in the drawing (B).

two proteins present in active enzyme prepared from human foetal and monkey livers. This protein was also demonstrated in livers obtained from the patients who died of unrelated diseases (whose livers were normal). Study of this protein, and of fragments of the native enzyme which seem to co-purify, may be useful in the elucidation of the defect in PKU. The general approach may be useful in the study of other inborn errors of metabolism in which the enzyme involved is rapidly inactivated after death or when the mutant enzyme is inactive.

Monkey liver phenylalanine hydroxylase was purified as already described⁴. The small modifications (Fig. 1) which were made for crude extracts of human livers did not alter the proteins isolated. The specific activity of each preparation is shown in Fig. 1. The proteins thus purified were then studied by the Gradiopore technique⁶ modified by the use of sodium dodecyl sulphate (SDS) (Fig. 1).

Purified monkey liver enzyme yielded two proteins of similar molecular weight (Fig. 1*b* and *g*) which ran between the two marker proteins which were used (catalase molecular weight 57,500 and ovalbumin molecular weight 45,000) (Fig. 1*a*). This indicates molecular weights very similar to those found when enzyme purified from rat liver by traditional methods was analysed by SDS electrophoresis—two proteins with molecular weight of 50,000 and 49,000 respectively have been described⁷.

The preparation from the 16-week foetus showed a similar pair of proteins (Fig. 1*c*). The preparation from the 34-week foetus (Fig. 1*f*) from the normal human liver (Fig. 1*e*) and from the PKU liver (Fig. 1*d*) all showed only the protein of smaller molecular weight (protein 2). Additional bands corresponding to smaller protein species were also noted in the preparations from PKU liver, from normal livers, and from the 34-week foetal liver (Fig. 1*d-f*).

Phenylalanine hydroxylase should be the only protein in liver which can bind to the pteridine adsorbent at the high concentration of NaCl (0.8 M) used and specifically eluted by phenylalanine. Consequently there is good reason to believe that the protein purified by this technique represents phenylalanine hydroxylase in an active or inactive form. Material isolated from monkey liver and from the foetal livers showed substantial phenylalanine hydroxylase activity. One protein species (protein 2) purified from the normal human livers and from the PKU liver behaved in the same way as one of the proteins obtained from those livers with enzyme activity, and we believe that this protein represents a component of phenylalanine hydroxylase. This is supported by the close correspondence between the behaviour of the monkey liver enzyme purified by this method and that described for purified rat liver phenylalanine hydroxylase⁷.

Kaufman⁷ has produced smaller species which are still active, by proteolytic cleavage of purified rat liver phenylalanine hydroxylase. This type of molecule may be represented among the smaller species purified from human livers (Fig. 1*d-f*).

Protein 1 was found only in the active preparations from monkey liver and from the 16-week foetus. Rapid degradation after death may explain the absence of this protein from the 34-week foetal liver, and from livers obtained at autopsy from older patients which had low activity. The degradation of this protein species may explain the rapid loss in enzyme activity observed after death³. The same mechanism may explain the absence of this band from the PKU liver preparation. It is also possible, however, that it represents the effect of the PKU mutation.

The next step must be to study the structure of the proteins isolated from this PKU liver. The preparations analysed in the experiment illustrated in Fig. 1 have been subjected to isoelectric focusing. But because of the considerable heterogeneity revealed, it was not possible to detect a simple charge-change mutation.

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Inhibition of reverse transcriptases of primate type C viruses by 7S immunoglobulin from patients with leukaemia

WE have characterised¹ type C virions which are released spontaneously from a diploid strain of human embryonic fibroblasts. These 'HEL-12 virions' have structural and biochemical properties of known RNA tumour viruses and are antigenically related to subhuman primate type C viruses. Immunological analyses have so far excluded cross reactivity between HEL-12 virions and avian (ASV), murine MuSV, or feline RNA sarcoma or leukaemia viruses.

Several lines of evidence strongly suggest that HEL-12 virions originate in the human cell strain from which they were isolated¹. Unambiguous proof of their human origin is, however, difficult to obtain, particularly in the absence of an undisputed human type C reference virus. We have approached this problem by asking if humans have humoral immunity to HEL-12 virion antigens. Because antibody to RNA-dependent DNA polymerase (reverse transcriptase²) is easily quantifiable and can be used to distinguish primate from non-primate type C viruses³, purified immunoglobulin (Ig) from eight patients with various types of leukaemia was assayed for anti-reverse transcriptase antibody. We found that the 7S Ig from the plasma of two leukaemic patients significantly inhibited the incorporation of ³H-dTTP by the reverse transcriptase of HEL-12 virions, woolly monkey fibrosarcoma (simian sarcoma) virus (SiSV)⁴ and baboon endogenous virus (BEV)⁵.

Ig was purified from the plasma of eight patients with leukaemia by differential fractionation with ammonium sulphate and rate zonal sedimentation of the precipitated Ig in 10–40% linear sucrose gradients⁶. The major protein peak migrated with a sedimentation coefficient of 7S (Fig. 1). The gradient profiles of Ig from leukaemic patients were not substantially different from those obtained with Ig from normal adult volunteers (Fig. 1). The pooled, purified 7S fraction from each patient was dialysed exhaustively, and antibody was quantitated by radial immunodiffusion in Agarose plates embedded with rabbit anti-human IgG, IgA or IgM (ref. 8). All precipitated protein was accounted for as either IgG (~90%) or IgA (~10%); no IgM was detected. Electrophoresis in sodium dodecyl sulphate (SDS)-polyacrylamide gels⁹ revealed that

Table 1 Inhibition of type C virus reverse transcriptases by purified 7S Igs from eight leukaemic patients

Patient	Haematological diagnosis	Maximal Ig concentration (μg)	Maximal inhibition (%) of reverse transcriptases						SR-RSV
			HEL-12	HL32V-1	BEV	SiSV	G-MuLV	KiMuSV (KiMuLV)	
N.K.	ALL	56	<10	<10	<10	<10	<10	13	<10
J.J.	ALL	60	<10	12	13	<10	<10	<10	<10
D.W.	CML	63	<10	ND	<10	<10	<10	<10	<10
E.P.	AML	76	<10	<10	<10	<10	<10	ND	12
T.K.	AML	80	<10	<10	12	<10	<10	<10	<10
B.J.	AML	72	<10	12	<10	<10	<10	<10	<10
M.R.	AML	64	53	11	72	42	18	<10	<10
R.M.	AML	70	66	37	81	32	<10	12	<10

Ig was purified from plasma of each patient as described in Fig. 1. Growth and purification of HEL-12 virions, G-MuLV, and Kirsten murine sarcoma (leukaemia) virus (KiMuSV (KiMuLV)) described previously¹⁻⁹. BEV, gift of Dr R. C. Gallo, National Cancer Institute, Bethesda, Maryland. HL23V-1, type C particle originally isolated by Gallagher and Gallo¹¹ now grown in dog thymus cells¹². SiSV and Schmidt-Ruppin strain of Rous sarcoma virus (SR-RSV), (Electro-Nucleonics Laboratories, Bethesda, Maryland) were disrupted and frozen immediately on receipt¹. To test for Ig inhibitory activity, all viruses were disrupted for 5 min at 37 °C in 0.1% Nonidet P40 (NP40) detergent; 5 μl virus ($\sim 12,000$ –80,000 c.p.m. ³H-dTTP rendered acid precipitable in 40 min using poly(A)-oligo(dT)₁₂₋₁₈ as template) were mixed with 5 μl 0.6 M KCl solution containing 50 mg ml⁻¹ bovine serum albumin (KCl-BSA). To this was added 20 μl dilution of appropriate Ig. Entire mixture incubated for 2 h at 4 °C; 170 μl reverse transcriptase reagents (0.025% NP40, 25 mM Tris-HCl, pH 8.1, 7m M 2-mercaptoethanol, 0.2 mM MnCl₂, 60 mM KCl, 1 μg poly(A)-oligo(dT)₁₂₋₁₈ (Collaborative Research, Waltham, Massachusetts) 5 μCi ³H-dTTP (specific activity 55 Ci mmol⁻¹, Schwarz-Mann, Orangeburg, New York) and 0.0083 mM 'cold' dTTP (Sigma) were added; entire mixture incubated at 37 °C for 40 min. Reaction mixture chilled quickly in ice and precipitated by addition of 25 volumes ice-cold 5% TCA–2% sodium pyrophosphate. Acid-precipitable radioactivity determined^{9,13}. Inhibition expressed relative to reactions containing virus without Ig. This reference value determined from average of four identical reaction mixtures. Figures represent percentage inhibition of reverse transcriptase at highest Ig concentration used. In cases in which inhibition was observed, value given represents a plateau level, at which increasing the Ig concentration resulted in no further inhibition of enzymatic activity.

ALL, Acute lymphocytic leukaemia; CML, chronic myelocytic leukaemia; AML, acute myelocytic leukaemia; ND, not determined.

more than 95% of the purified protein migrated, as expected for heavy and light chains of IgG and IgA (ref. 10).

The extent of inhibition of the reverse transcriptase from various type C viruses was compared using purified 7S Ig from each patient (Table 1). Ig from six of the patients did not inhibit significantly the reverse transcriptase of any of the viruses tested. 7S Ig from patients M.R. and R.M., however, reduced the incorporation of ³H-dTTP by reverse transcriptase in the following order: BEV>HEL-12>SiSV>HL23V-1 (ASV7573), (Table 1 and Fig. 2). These findings were confirmed repeatedly for Ig fractions obtained at different times from each of the patients. The difference in inhibition between the enzymes from HEL-12 virions and SiSV is probably related to the antigenic heterogeneity of the former¹⁴. We have found¹⁴ that the HEL-12 virion population contains at least two components, one with antigenic characteristics of SiSV, the other with properties of BEV. Interestingly enough, the reverse transcriptase of a type C virus isolated by Gallagher and Gallo^{11,12} and referred to as HL23V-1, was not significantly inhibited by 7S Ig from patient M.R. The reasons for the lack of reverse transcriptase inhibition of this isolate are unclear, even though the HL23V-1 virus is known to be related to SiSV. The results may be attributable to qualitative differences between the 7S Ig fractions of patients M.R. and R.M., as

well as antigenic differences between the reverse transcriptases of HEL-12, HL23V-1 and primate viruses such as SiSV. We have also examined for inhibitory activity the 7S Ig fraction of six normal volunteers. The viruses used as sources of reverse transcriptase included HEL-12, SiSV,

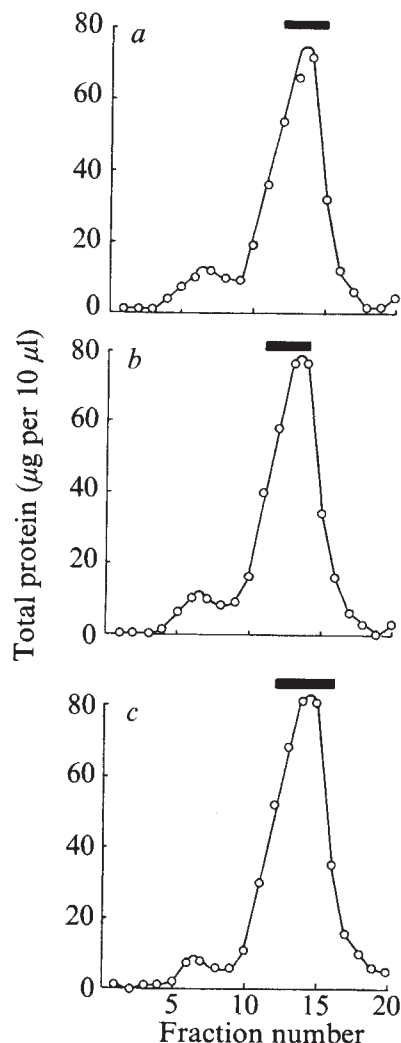


Fig. 1 Representative zonal sedimentation profiles of Ig from patients M.R. (a) and R.M. (b), and a normal volunteer (c). Blood from patients drawn into sterile, heparinised tubes, allowed to stand at 4 °C for 3–8 h and centrifuged at 1,100 r.p.m. for 10 min. Plasma stored at –20 °C. For Ig purification, 1.0 ml plasma from each patient was diluted with an equal volume of TNE buffer and slowly mixed with 2.0 ml saturated solution ammonium sulphate at 4 °C. After stirring for 30 min, precipitate centrifuged for 30 min at 2,000g. Supernatant fluid discarded, pellet resuspended in 2.0 ml TNE buffer, and precipitation by ammonium sulphate repeated. After centrifugation at 2,000g, pellet resuspended in 300 μl TNE buffer and dialysed exhaustively at 4 °C against 0.04 M phosphate buffer, pH 6.9. 300 μl dialysate layered on 5 ml linear 10–40% sucrose gradient in TNE buffer and centrifuged at 4 °C in a SW50.1 rotor at 35,000 r.p.m. for 16.25 h. 20 equal-volume fractions collected from bottom of tube, and 10 μl aliquots assayed for protein according to method of Lowry *et al.*⁷. IgG purified on Sephadex G-200, included in a parallel gradient, sedimented at fraction 14. Fractions indicated by bars were pooled, dialysed exhaustively against 0.1 M Tris-HCl, pH 8.0, and stored at –20 °C. Direction of sedimentation from right to left.

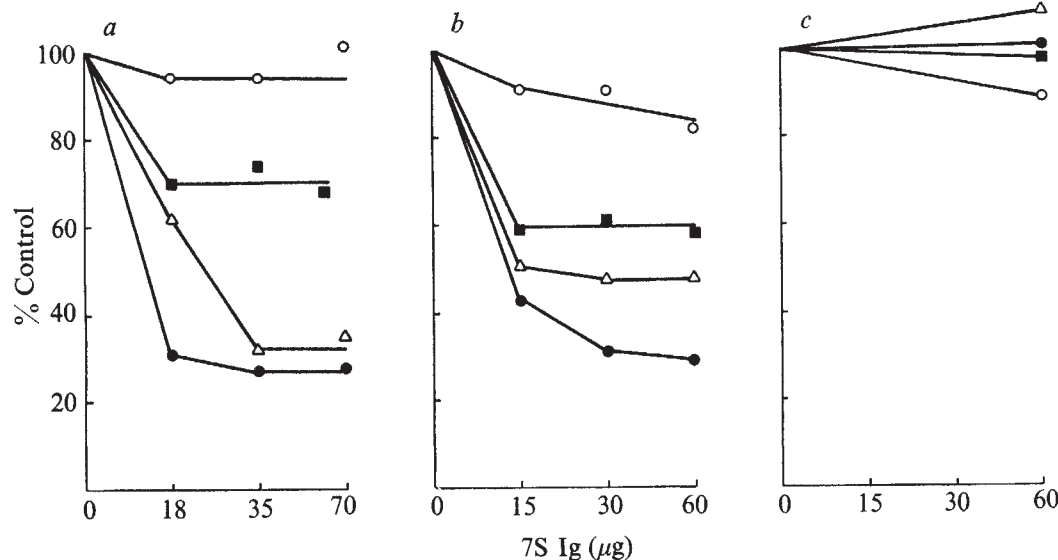


Fig. 2 Inhibition of type C virus reverse transcriptases by purified 7S Ig. 20 μ l dialysed 7S Ig fraction from patient M.R. (a) or R.M. (b) or from a normal volunteer (c) incubated for 2 h at 4 °C with 5 μ l KCl-BSA and 5 μ l appropriate NP40-disrupted type C virus (12,000–80,000 c.p.m. 3 H-dTTP incorporated in 40 min). Reverse transcriptase reagents added (170 μ l). Acid-precipitable 3 H-radioactivity measured after 40 min incubation at 37 °C. All values expressed relative to average of quadruplicate reactions for each virus run in absence of 7S Ig. Each point represents average of duplicate samples. $\circ$, G-MuLV; $\blacksquare$, SiSV; $\triangle$, HEL-12 virions; $\bullet$, BEV.

and Gross murine leukaemia virus (G-MuLV). In none of the cases was a greater than 10% inhibition observed at comparable 7S Ig concentrations (not shown).

The above observations with the 7S Ig of patients M.R. and R.M. were extended to the purified reverse transcriptases of avian myeloblastosis virus (AMV) and SiSV. At the concentrations tested, 7S Ig from patients M.R. and R.M. did not significantly inhibit the activity of AMV reverse transcriptase (Fig. 3). Substantial inhibition of the SiSV enzyme was, however, observed in the case of both Igs.

Since destruction of the RNA template or of the enzymatic poly(dT) product by plasma nucleases could account for the observed inhibition, 7S Igs were incubated with 3 H-poly(A) or with poly(A)- 3 H-poly(dT) (Table 2). As determined by hydrolysis of the labelled polynucleotides, Ig from leukaemic patients did not contain poly(A) nuclease activity nor was 3 H-poly(dT) hydrolysed to a significant degree by any of the Igs tested.

The inhibition might also have been due to the presence of anti-nucleic acid antibodies in the Ig fraction. Aliquots of Ig were therefore incubated with 3 H-poly(A) or with poly(A)- 3 H-poly(dT). The Igs were precipitated with half saturated ammonium sulphate which precipitates only

nucleic acid molecules complexed with antibody¹⁷. As shown in Table 2, anti-nucleic acid antibodies were not detected by this assay method in any of the samples tested.

Why do patients with leukaemia develop a humoral antibody against an internal virion component such as reverse transcriptase? Three explanations can be offered: leukaemic leukocytes may produce type C viruses which are degraded and release antigenic reverse transcriptase^{11,18,19} related to the reverse transcriptase of HEL-12 virions; lysis of leukaemic leukocytes may release "particle-associated reverse transcriptase"^{20–22} (note that both patients with inhibitory antibody had undergone long term chemotherapy and had survived for approximately one year from the time of initial diagnosis; however, the patients were never in haematological remission); reverse transcriptase may be exposed at the surface of leukaemic leukocytes, as are other internal virion proteins in infected animal cells^{23–25}.

Irrespective of the mechanism by which reverse transcriptase exerts its antigenic effect, the antibody response observed in the two patients seems highly specific for the reverse transcriptases from viruses of primate origin. The specificity of reverse transcriptase inhibition was substantiated using purified enzyme preparations derived from stocks of AMV and SiSV. Thus, it cannot be argued that

Table 2 Nucleases and anti-nucleic acid antibodies in 7S Ig from leukaemic patients

Patient	7S Ig (μ g)	Nuclease activity*, 3 H-poly(A)	% hydrolysis of poly(A)- 3 H-poly(dT)	Anti-nucleic acid antibody†, 3 H-poly(A)	% precipitated poly(A)- 3 H-poly(dT)
N.K.	28	13.1	4.0	<0.1	11.2
J.J.	30	–6.4	–13.0	0.1	7.9
D.W.	32	20.0	–10.7	<0.1	7.2
E.P.	38	–2.8	5.4	0.1	6.3
T.K.	40	7.6	4.5	0.1	5.8
B.J.	36	–8.2	–15.1	<0.1	12.8
M.R.	32	1.5	4.2	<0.1	13.5
R.M.	35	–10.9	–4.4	<0.1	5.8

* 3 H-poly(A), Miles Laboratories, Elkhart, Indiana. Poly(A)- 3 H-poly(dT), synthesised in a standard reverse transcriptase reaction^{1,9,13} using purified reverse transcriptase from AMV as enzyme source (Schwarz-Mann). Reaction mixture diluted tenfold in TNE buffer (0.1 M NaCl, 10 mM Tris-HCl, pH 7.3, 1 mM EDTA) and extracted once with TNE-saturated recrystallised phenol and once with chloroform-isoamyl alcohol (24:1). Product precipitated at –20 °C for 48 h by addition of 2 volumes 100% ethanol. After centrifugation at 10,000g for 60 min, pellet resuspended in a minimal volume 0.1 M Tris-HCl, pH 8.0, and stored at –20 °C. After isopycnic centrifugation in neutral CsCl gradients, > 95% 3 H-radioactivity found associated with RNA.

†Hydrolysis by 7S Ig measured after incubation of 10 μ l Ig and 5 μ l 3 H-poly(A) (~1,500 c.p.m. per 5 μ l) or poly(A)- 3 H-poly(dT) (~1,000 c.p.m. per 5 μ l) for 2 h at 4 °C and for 40 min at 37 °C. Mixture precipitated by addition of excess ice-cold 10% TCA. Acid-insoluble material collected on a 0.45- μ m membrane filter (Millipore) and dried thoroughly. Unhydrolysed radioactivity measured in toluene-based scintillation fluid.

‡10 μ l Ig in 0.1 M Tris-HCl, pH 8.0, incubated with 5 μ l 3 H-poly(A) or poly(A)- 3 H-poly(dT) for 2 h at 0 °C and for 40 min at 37 °C. Ig precipitated by addition of 5 ml half-saturated ammonium sulphate solution. Precipitate centrifuged, washed with additional 5 ml half-saturated ammonium sulphate solution and centrifuged. Amount of 3 H-label in precipitate determined and expressed as percentage total acid-precipitable input c.p.m.

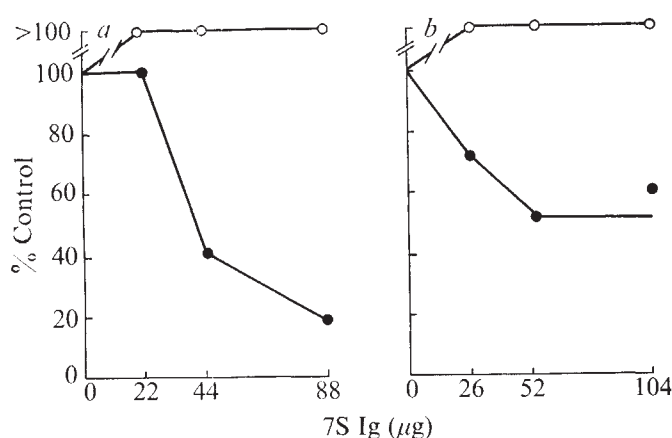


Fig. 3 Inhibition of purified reverse transcriptases by 7S Ig from patients M.R. (a) and R.M. (b). AMV (○) and SiSV (●) reverse transcriptases purified as described previously^{15,16}. AMV enzyme, purchased from Schwarz-Mann; SiSV enzyme, gift of Drs R. E. Gallagher and R. C. Gallo. Both enzymes stored at -20°C in 0.26 M KCl, 30 mM Tris-HCl, pH 7.9, 0.6 mM dithiothreitol, 0.06% Sterox SL, and 50% glycerol. 40 μl 7S Ig in 0.1 M Tris-HCl, pH 8.0, incubated with 10 μl enzyme for 2 h at 4°C with constant mild agitation. Reverse transcriptase reagents (150 μl) were added (see Table 1) and mixture incubated for 40 min at 37°C . Each point determined from average of triplicate samples and expressed relative to average of six identical control samples containing enzyme in absence of 7S Ig.

the selective inhibition was the result of differential accessibility of the reverse transcriptases of primate viruses or of a nonspecific inhibition. The results indicate that some adult patients with leukaemia mount a humoral immune response against two antigenically distinct reverse transcriptase or against an enzyme which shares determinants with both SiSV and BEV enzymes. Although our observations are consistent with findings of SiSV- and BEV-related polypeptides in certain human tissues^{20,22,26-28}, our patient population is obviously too small to conclude that the humoral response to reverse transcriptase is confined strictly to patients with leukaemia.

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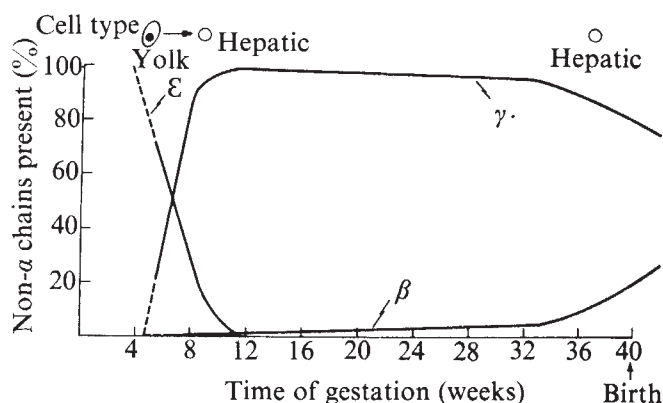
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Increasing haemoglobin β -chain synthesis in foetal development is associated with a declining γ - to α -mRNA ratio

DURING human foetal development, the predominant haemoglobin in erythrocytes switches twice (Fig. 1). First, ϵ chains of embryonic haemoglobins in erythroblasts of yolk sac origin are replaced by γ chains of foetal haemoglobin and traces of β chains of adult haemoglobin. This switch occurs very early in foetal development, coincident with the appearance of a new type of peripheral erythrocyte of hepatic origin. Second, at about 32 weeks of gestation, predominantly γ -chain production gives way to increased β -chain synthesis within a constant cell type¹. This double haemoglobin switch also occurs in other animals, including sheep². Not only is very little known about the biological basis for the switch from γ - to β -chain production, but the basic changes in gene activity involved in the switching process are obscure. An understanding of the γ to β switch has clinical importance, for if one could block the switch and increase γ -chain production, sickle cell anaemia and β -thalassaemia could be treated effectively. We present here data concerning changes in globin gene activity, as assessed by globin mRNA levels, which are temporally related to the turning up of β -chain synthesis. These observations were originally made in erythroid cells of aborted human foetuses and were extended to cells from foetal sheep.

Globin mRNA of human sheep reticulocytes was isolated by oligo(dT)-cellulose chromatography of polyribosomal RNA^{3,4}. Gould and Hamlyn demonstrated that the mRNA region of rabbit reticulocyte RNA contained two bands after acrylamide gel electrophoresis in formamide⁵, and subsequent work in many laboratories demonstrated that the faster migrating of these RNAs is α mRNA and the slower is β mRNA⁶⁻¹⁰. This electrophoretic procedure separates the

Fig. 1 Percentage of non- α chains in foetal development plotted against time of gestation. The erythroid cell type, yolk sac or hepatic, is shown above.



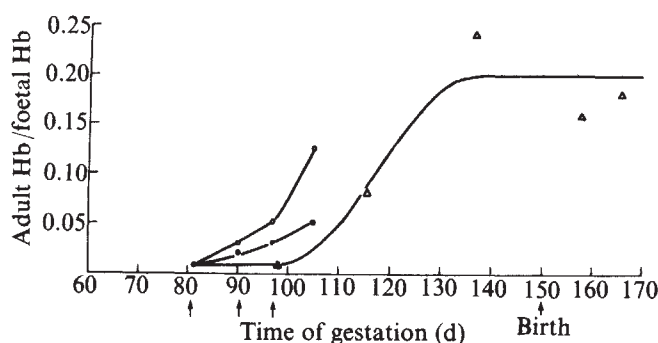


Fig. 2 Synthesis of adult haemoglobin (Hb) compared with that of foetal haemoglobin plotted against age of sheep from conception. The normal curve (Δ) is shown in addition to data obtained on two foetal sheep bled at times indicated by the arrows. $\circ$, Sheep 5,283; $\bullet$, sheep 5,216. Erythroid cells of foetal sheep were washed and incubated with ^{35}S -methionine for 3 h (ref. 15). The haemoglobins were separated by Bio-Rex 70 chromatography as described for separation of human foetal and adult haemoglobins¹⁵ except that the starting buffer was 0.019 M in sodium phosphate. Sheep haemoglobins A and B elute close to one another with starting buffer at 25 °C, and haemoglobin C elutes when the sodium phosphate concentration is increased to 0.058 M at 25 °C. Haemoglobin C synthesis was questionable except in sheep 5283 at 105 d when it accounted for nearly 5% of total haemoglobin synthesis.

α and β mRNAs in several species, including man^{10,11}, mouse⁷, rabbit⁶, sheep and goat (unpublished results of H.H.K. and P.G.S.). The same technique has shown that β mRNA is deficient in reticulocyte RNA preparations from human foetuses¹¹, and that in man, γ mRNA has an intermediate mobility between the β and α mRNAs¹⁰⁻¹¹.

In globin mRNA preparations from human adult reticulocytes, the ratio of β mRNA to α mRNA was determined by gel electrophoresis to be near 1 (ref. 11). Unfortunately, we have been unable to separate the γ and α mRNAs sufficiently to allow accurate estimation of the ratio of their concentrations in foetal cells.

Therefore, we assayed foetal mRNA preparations in an ascites cell-free system¹² and compared the γ - to α -chain

synthesis ratios with the β to α ratios obtained with mRNA from adult cells (Table 1). Although the cell-free system does not facilitate quantitation of the absolute ratios of chains synthesised, it makes possible the comparison of chain synthesis ratios directed by different mRNA preparations when mRNA is present in limiting concentrations. In this system the RNA from sickle cell patients and a 32-week premature infant gave β/α or γ/α ratios near unity. In contrast, assays of mRNA from mid-trimester foetuses

Table 1 Cell-free globin synthesis ratios

Human mRNA source	Chain synthesis ratio
	(β/α)
Adults (SS disease)	1.1
	1.0
	(γ/α)
32-week premature infant	1.4
Mid-trimester foetuses	4.0
	4.5

The source of globin mRNA for these assays was reticulocyte polyribosomal RNA. Assay conditions were those previously described¹² and mRNA concentrations were less than 1 μg per 60 μl , well below the concentration of 4–6 μg mRNA per 60 μl , which is non-limiting in our laboratory. Product was analysed by chain separation on Cellogel electrophoresis¹⁶. In addition, a γ/α -synthetic ratio of 2.1 was obtained in a mid-trimester foetal sample analysed by peptide mapping which is twice the β/α ratio observed in assays of adult mRNA preparations analysed in this manner.

resulted in γ/α -chain ratios much greater than 1. Thus, cell-free assays suggested that γ/α -mRNA ratios were greater than 1 at mid-trimester and near 1 at 32 weeks of gestation in man.

To provide further evidence for a declining γ/α -mRNA ratio in late foetal development, we used cells from foetal sheep. This animal has the following advantages. (1) Haemoglobin switching during development is very similar to that in man. At birth, adult haemoglobin accounts for 5–10% of total haemoglobin². (2) Foetal sheep can be obtained at known gestational ages. (3) The foetus can be removed surgically from the uterus, manipulated and returned to the uterus without significant mortality.

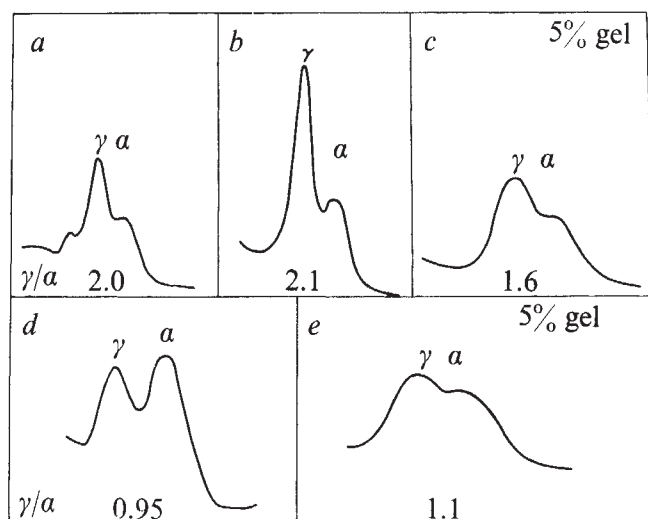
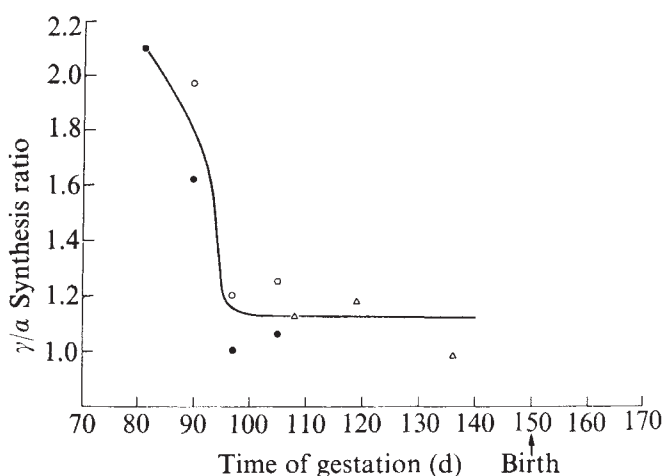


Fig. 3 Gel scans of globin mRNAs of foetal sheep at: a, 74 d; b, 81 d; c, 95 d; d, 98 d; e, 115 d of gestation. Reticulocytes were washed and lysed, and polyribosomes were obtained by ultracentrifugation⁸. The RNA solution was extracted, chromatographed on oligo(dT)-cellulose⁴, and the mRNA fraction was subjected to electrophoresis in formamide on 7% polyacrylamide gels⁹. Scans of the gels after treatment with Stains-All were performed at 600 nm. Results shown in (c) and (e) were obtained with 5% polyacrylamide gels.

Fig. 4 The γ/α synthesis ratio of foetal sheep reticulocytes plotted against time of gestation. Reticulocytes were incubated with ^3H -leucine and the radioactive chains were separated by Cellogel electrophoresis in urea and mercaptoethanol¹⁶. The relative amounts of γ and α chains recovered after electrophoresis and staining were determined by the absorbancy of dissolved Cellogel strips and the data were used to correct the d.p.m. associated with the γ and α chains for the yield of each chain. Results obtained with sheep foetuses that were not chronically bled are indicated by triangles. Bled foetuses were: $\bullet$, 5,283; $\circ$, 5,216.



Blood samples were obtained from foetal sheep at various times during gestation, and haemoglobin synthesis, together with globin mRNA quantities, was studied in the cells. The ratio of adult haemoglobin synthesis to foetal haemoglobin synthesis was determined at various times of gestation (Fig. 2). The synthesis of adult haemoglobin is about 1% that of foetal haemoglobin until about 100 d of foetal life, which is two-thirds of the way through gestation. It then rises to 20% that of foetal haemoglobin by 130–140 d of gestation.

When globin mRNA of foetal sheep is subjected to formamide gel electrophoresis, unlike the situation in man, the non- α mRNA (the presumed γ mRNA) separates quite well from α mRNA (Fig. 3). When these two RNAs were eluted separately from gels in which the RNA separation was suboptimal, the slower migrating band directed the synthesis of 1.6 times as many γ chains as α chains in the cell-free system, while the faster migrating band directed the synthesis of a twofold excess of α chains. Sheep β mRNA has an electrophoretic mobility similar to that of γ mRNA by this technique. But β -chain synthesis accounts for less than 10% of non- α chain synthesis up to 115 d of gestation, indicating that γ mRNA should be free of significant β -mRNA contamination until late in gestation. The ratio of γ mRNA to α mRNA was about 2 in reticulocytes at 70–80 d and then declined to near 1 at 98–100 d of gestation. Although it remained in this general range, a sample obtained at 145 d had a (γ mRNA + β mRNA) to α mRNA ratio of 1.3.

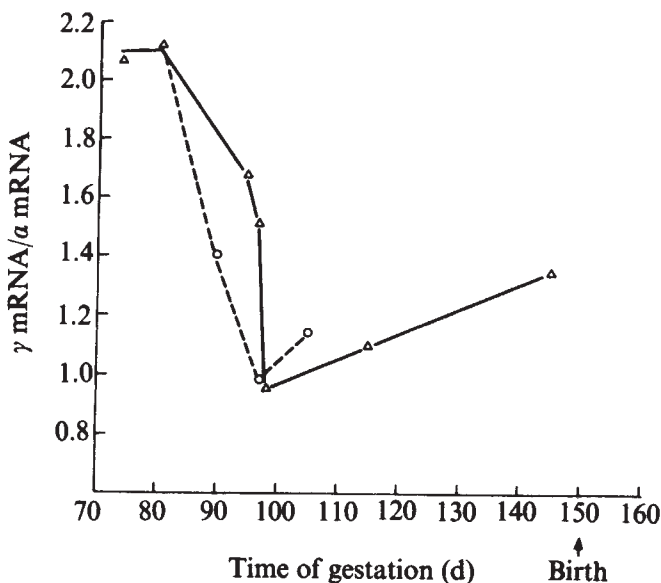


Fig. 5 The γ/α -mRNA ratio plotted against the time of gestation. Results from normal (Δ) and chronically bled foetuses ($\circ$) are shown. Blood from the two foetuses undergoing bleeding was pooled at each point to ensure sufficient mRNA for analysis.

To determine whether the decline in γ/α -mRNA ratio is important for the subsequent turning up of β -chain synthesis, we attempted to alter the timing of increased β -chain synthesis by chronic bleeding of foetal sheep. Two foetuses were bled of approximately one-fifth of their blood volume (40 ml) at 81, 90 and 97 d, and then bled and killed at 105 d of gestation. Haemoglobin and globin chain synthesis by reticulocytes, and globin mRNA levels were studied at these times. In one bled foetal sheep, the synthesis of adult haemoglobin increased prematurely, reaching 12.5% that of foetal haemoglobin at 105 d of gestation (Fig. 2). Adult haemoglobin synthesis in untreated foetuses at that gestational age is about 3% of foetal haemoglobin synthesis. In the second sheep, adult haemoglobin synthesis was also

increased, but the moderate rise observed may not be significantly different from normal.

We then asked whether the premature increase in β -chain synthesis was associated with premature changes in γ/α mRNA and γ/α -chain synthesis ratios. In Fig. 4 the reticulocyte γ/α -chain synthesis ratio is plotted against the time of gestation in the bled foetuses. This ratio was about 2 at 80 d of gestation, suggesting that in the sheep translational controls do not operate to balance the synthesis of α and γ chains in the early foetus. In contrast, synthesis of α and γ chains during early foetal life in man is balanced¹³. Moreover, the γ/α -chain synthesis ratio dropped to 1 at about 90–100 d of gestation. Just as the ratio of γ/α -chain synthesis declined at 90 d of gestation, so the γ/α -mRNA ratio also declined precipitously between 90 and 100 d of foetal age in both normal foetuses and bled foetal sheep (Fig. 5).

These studies of the turning up of β -chain synthesis suggest the following conclusions. First, β -chain switching in the sheep may be hastened by continued bleeding of the foetus. Whether this premature turning up of β -chain synthesis is a response to stress, hypoxia or other factors is not clear. Erythroprotein was not detectable in the blood of the bled foetuses until the day of death when it was 0.2 U ml^{-1} in one sheep (denoted 5,283, data of J. L. Spivak). Second, the γ/α -mRNA ratio is greater than 1 early in foetal development and decreases to unity around the time of increased β -chain production.

These data and the fact that the number of γ genes in man is at least twice that of β genes^{1,14}, suggest the following hypotheses. (1) Active γ -chain genes lead to a γ/α -mRNA ratio of 2 in the foetus, in contrast to the β/α -mRNA ratio of 1 in the adult. (2) This ratio is maintained until about 30 weeks of gestation in man and 90 d of gestation in sheep when transcription of γ -chain genes declines, leading finally to a partial deficiency of γ chains within precursor erythroid cells, which is followed by an increased production of β chains in these cells. The data presented here indicate that a relative decline in γ mRNA occurs in reticulocytes at about the time of increased β -chain synthesis, but they do not demonstrate that this decline is a necessary precedent to the turning up of β -chain production. In these experiments a premature increase of β -chain synthesis was produced in two bled foetal sheep, but a premature decline in the γ/α -mRNA ratio and the parallel γ/α -chain synthesis ratio, which the data suggest, requires further investigation.

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Half life of yeast messenger RNA

We have determined the half life of *Saccharomyces cerevisiae* messenger RNA (mRNA) and its associated poly(A) sequences, from the kinetics of their labelling with ¹⁴C-adenine. Corrections have been made for changes in the ATP pool specific activity during the labelling period. This method of determining the mRNA half life is preferred to observing the decay of mRNA in the presence of inhibitors because changes in the cell metabolism caused by an inhibitor may also change the mRNA half life¹. One of our purposes in determining these half lives is for their use in the characterisation of temperature-sensitive mutants which are defective in macromolecular synthesis². For this reason we have determined the half lives of mRNA and poly(A) in two different conditions, at 23 and 36 °C.

The incorporation of ¹⁴C-adenine into mRNA has been followed in total mRNA and in the poly(A) portion of mRNA. The total mRNA was obtained from polysomes isolated on sucrose gradients. To separate rRNA and mRNA, the polysome fractions were collected and rerun on 10–30% sucrose gradients in low magnesium buffer, in which the polysomes dissociate into 40S and 60S subunits and mRNA. Figure 1 shows a typical subunit gradient, in this case from polysomes labelled for 30 min at 36 °C with ¹⁴C-adenine. The rRNA in the 60S and 40S subunits is clearly resolved. The mRNA forms a broad peak that, for the most part, sediments more slowly than the 40S subunit. The division between counts in mRNA and subunits is estimated by assuming that the specific activity of the subunit RNA is constant across the peak. (The tRNA accounts for < 3% of the radioactivity in the

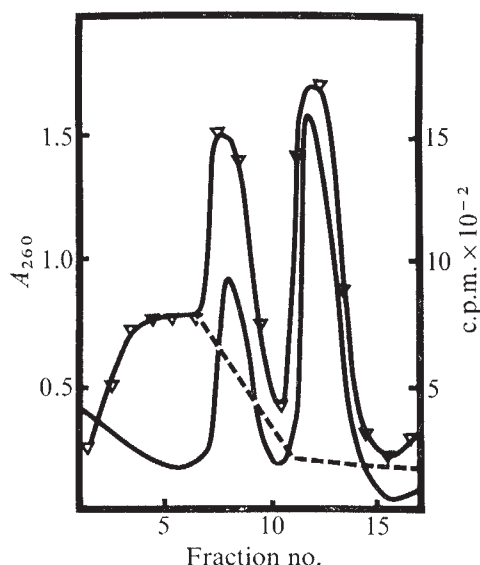


Fig. 1 Dissociation of polysomes into ribosomal subunits and mRNA. Cells were converted to spheroplasts¹², which were labelled with ¹⁴C-adenine for the indicated time, lysed, and layered on sucrose gradients containing 0.03 M Mg²⁺ (ref. 12). The polysome region (as determined by an ultraviolet scan) from these gradients was pooled and centrifuged at 160,000g for 3 h and the pellet resuspended in S buffer (0.01 M Tris, 0.1 M NaCl, 10⁻³ M MgCl₂, pH 7.4) to dissociate the polyribosomes. This was layered on a 15–30% sucrose gradient in S buffer and centrifuged at 90,000g for 17 h. The gradient shown is representative, and is from a culture labelled for 30 min at 36 °C. ▽, c.p.m. in ¹⁴C-adenine. The theoretical curves for mRNA (---) and rRNA (—) are also shown.

Table 1 Ratio of ³H/¹⁴C in intercellular pools*

Time labelled with ³ H-adenine (min)	ATP			
	23 °C	36 °C		
Average value	Mean deviation	Average value	Mean deviation	
5	0.41	0.01	0.38	0.04
15	0.83	0.12	0.88	0.01
30	1.00		1.00	

*Spheroplasts were prepared as in Fig. 2 and allowed to grow for 3 h in media labelled with 0.5 μCi ml⁻¹ ¹⁴C-adenine (specific activity 45 mCi mmol⁻¹). 30 μCi ml⁻¹ ³H-adenine (10 Ci mmol⁻¹) was added, and 1-ml samples were collected in equal volumes of 10% TCA. The TCA samples were filtered, and the filtrate was charcoal adsorbed and washed as described in ref. 9, except that the mixture used to elute the nucleotides from the charcoal was ethanol–28% NH₄OH–H₂O (65:2:32 v/v). The samples were dissolved in water with carrier ATP, GTP, UTP, and CTP, and were spotted on thin-layer PEI cellulose plates (Brinkman, CEL 300 PEI, 20 × 20 cm). The solvent system used to separate the trinucleotides was developed by Neuhaed *et al.*¹⁰ and modified by Mowbray¹¹. Plates were developed 3 cm in 2 M LiCl–2 M acetic acid (1:1 v/v) and then to 15 cm in 2.5 M LiCl–2 M acetic acid (1:1 v/v) in the first dimension. They were dried, washed in methanol, and developed in the second dimension first to 5 cm in 3 M ammonium acetate, 3.6% boric acid pH 7.0 and then to 15 cm in 4 M ammonium acetate, 5% boric acid pH 7.0. The plates were dried and the ATP and GTP spots identified by autoradiography and the ultraviolet absorption of the markers. These spots were cut out and counted to determine the ³H/¹⁴C ratio.

ribosomal subunits.) The radioactivity in mRNA is determined from these gradients and normalised to the absorption in total ribosomal RNA on the gradients, to correct for variations in the extent of cell lysis in the sample. These data are plotted in Fig. 2. The curve drawn through the points is a computer fit of equation (3), as is discussed below.

The half life of poly(A) in polysomes was determined by following the incorporation of ¹⁴C-adenine into nuclease-resistant material, isolated from polysomes, which binds to poly(dT)–cellulose columns⁴. These data were fitted in the same way as total mRNA (Fig. 3).

Table 2 Decay constants of yeast RNA*

	mRNA			Poly(A)		
	μ	k	Half life	μ	k	Half life
23 °C	0.0416	0.115	16.6	0.0601	0.115	11.5
36 °C	0.0421	0.128	16.4	0.0547	0.128	12.6

*Values have been obtained from the computer fit of the data shown in Figs 2 and 3 and are corrected for the labelling of the ATP precursor. The values for the half lives of mRNA and the associated poly(A) sequences, uncorrected for the labelling kinetics of the ATP pool, were ~ 9 min longer in each case.

The radioactivity in mRNA as a function of time depends on the specific activity of the ATP precursor pools as well as the rate of synthesis and destruction of mRNA. If the change in specific activity of the ATP pools was neglected, the amount of mRNA after short times would be underestimated relative to the amount made after longer periods of labelling. We measured the specific activity of the ATP pool in cultures labelled identically to those used for the determinations of half life using two-dimensional thin-layer chromatography to isolate cellular nucleoside triphosphates (Table 1). Since the conversion of adenine to guanine is restricted by the presence of non-radioactive guanine in the medium, the labelling of GTP was ~ 10% of that of ATP. This yielded, given the relative sizes of the ATP and GTP pools³, a specific activity of the GTP of < 30% of that of the ATP at the longest labelling times. The evolution of this rise in specific activity, in so far as could be measured (data not shown), followed that of the ATP. For these reasons a correction for the kinetics of labelling of the GTP was not necessary.

We have fitted our data for the labelling of mRNA (Figs 2 and 3) to an equation which takes into account changes in ATP

specific activity. We assume that the specific activity of the mRNA is a function of the specific activity of the ATP pool and of the rate of synthesis and degradation of mRNA

$$\frac{dM}{dt} = \mu(P - M) \quad (1)$$

where M is the specific activity of the messenger pool, μ is the rate of synthesis of mRNA per unit of mRNA in the cell, and P is the specific activity of the ATP pool. The assumptions involved in this equation are that the amount of mRNA per unit cell mass is constant, and that the decay of mRNA is random. To obtain values for P we have fitted the data for the specific activity of the ATP pools to a curve of the form

$$P = 1 - \exp(-kt) \quad (2)$$

where k is the time constant of labelling of the ATP pool and the final specific activity of the ATP pool is defined as 1.0. Using equations (1) and (2) and solving for M

$$M = 1 + \left[\frac{k \exp(-\mu t) - \mu \exp(-kt)}{\mu - k} \right] \quad (3)$$

When the specific activity of the pool is constant, the solution to equation (1) is

$$M = 1 - \exp(-\mu t) \quad (4)$$

Table 2 gives the values of k and μ for each of the curves in Figs 2 and 3 as well as the half lives of the RNA. The half life of total mRNA is ~ 16 min at both 23 and 36 °C whereas that of poly(A) is ~ 12 min at both 23 and 36 °C. This difference of ~ 4 min in the half lives of mRNA and poly(A) could arise from: first, differences in the labelling of internal precursor pools from which poly(A) and mRNA are made;

Fig. 2 The kinetics of ^{14}C -adenine incorporation into mRNA. The curve is a least squares computer fit of the data points to equation (3) with k determined from the data in Table 1. The specific activity of mRNA is normalised to 100 for maximum labelling. *a*, mRNA labelled at 23 °C; *b*, mRNA labelled at 36 °C.

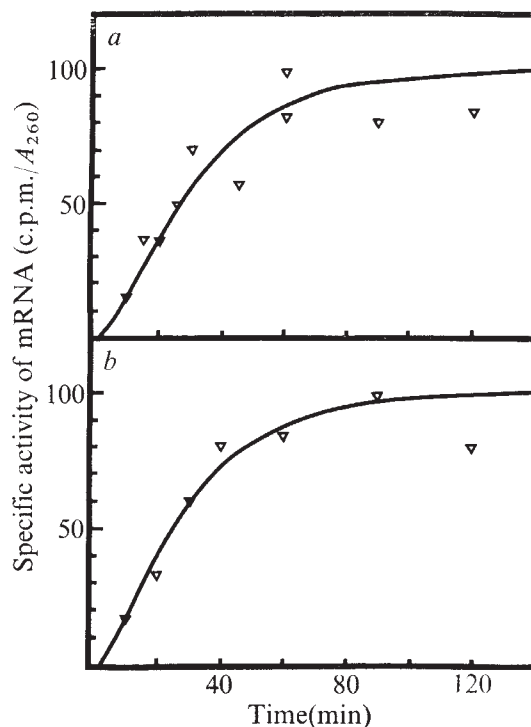
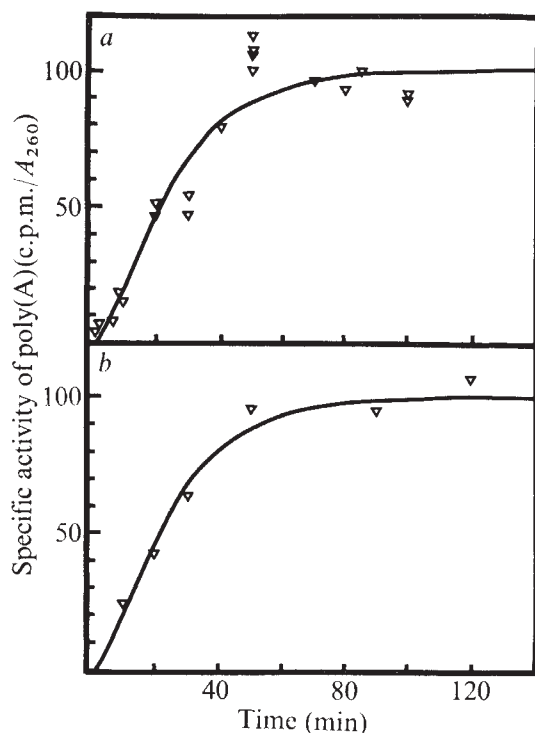


Fig. 3 The kinetics of ^{14}C -adenine incorporation into messenger-associated poly(A). The curve is a least squares computer fit of the data points to equation (3), with k determined from the data in Table 1. *a*, Poly(A) labelled at 23 °C; *b*, poly(A) labelled at 36 °C.

second, clipping of poly(A) during the lifetime of the messenger so that an older message would have shorter poly(A) sequences or third, the presence of a longer lived fraction of mRNA that contains no poly(A).

There have been several previous determinations of yeast mRNA half life. Hartwell *et al.* have measured the kinetics of incorporation of ^{14}C -adenine into polysomes in the presence of cyclohexamide and the decay of polysomes in the mutant *rna 1* which is defective in cytoplasmic RNA production⁵. Also, Tønneson and Friesen have followed the decay of protein synthesis in the presence of daunomycin or ethidium bromide⁶. The value obtained for the half life of mRNA in these experiments was ~ 22 min.

The value we obtained for the half life of the mRNA in yeast is 10–15% of the cell doubling time. This is intermediate between the values obtained for *E. coli* of ~ 3 –6% of the doubling time, and values obtained for HeLa cells and mouse C cells of 70% or more of the cell doubling time^{7,8}.

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Aminoacylation and nucleoside modification of *in vitro* synthesised transfer RNA

TRANSCRIPTION of transfer RNA (tRNA) genes *in vitro* by purified RNA polymerase has the advantage of producing completely unmodified tRNA precursors which provide a substrate for the study of the processes and modifications leading to the formation of mature tRNA molecules. We have shown¹ that the tRNA genes, *tRNA*₁^{Tyr} (*su*₃⁺ and *su*₃⁻), *tRNA*₂^{Gly} *su*⁺A36, *tRNA*₃^{Thr} and *tRNA*₂^{Tyr} (carried by the respective ϕ 80psu₃⁺ and λ h80T phage DNA^{2,3}), were transcribed *in vitro* by *Escherichia coli* RNA polymerase into polycistronic tRNA precursors. These precursors were cleaved on incubation with a supernatant fraction from *E. coli*, yielding mature size tRNA molecules¹. The transcription *in vitro* of an active *tRNA*₁^{Tyr} *su*₃⁺ by crude S-30 *E. coli* extracts or a combination of partially purified fractions has been described⁴⁻⁶. The formation of the primary transcription products of the *tRNA*^{Tyr} gene was not, however, clearly demonstrated and it was concluded that an additional *E. coli* extract fraction would be required to enable purified RNA polymerase to function in tRNA synthesis^{5,6}. We report here evidence indicating that the *in vitro* transcription of ϕ 80psu₃⁺ or λ h80T DNA by purified *E. coli* RNA polymerase alone produces tRNA precursor molecules which are not only processed to form mature-size tRNA¹ but are also recognised by specific modifying enzymes. In addition the tRNA molecules (tRNA₁^{Tyr}, tRNA₂^{Tyr}, tRNA₃^{Thr} and tRNA₂^{Gly}) synthesised and processed *in vitro*, possess amino acid acceptance activity.

For the study of the formation of modified nucleosides in *in vitro* synthesised tRNA, $\phi 80\text{psu}_3^{+/-}$ and λh80T DNA were transcribed by *E. coli* RNA polymerase in reaction mixtures containing $\alpha\text{-}^{32}\text{P}$ -CTP, $\alpha\text{-}^{32}\text{P}$ -UTP or $\alpha\text{-}^{32}\text{P}$ -ATP. After 30 min incubation at 37°C RNA synthesis was terminated by addition of actinomycin D and DNase I. Synthesised RNA was processed by incubation for 90 min at 37°C with a dialysed *E. coli* S-100 extract. RNA was extracted with phenol, precipitated using ethanol and fractionated by electrophoresis on a

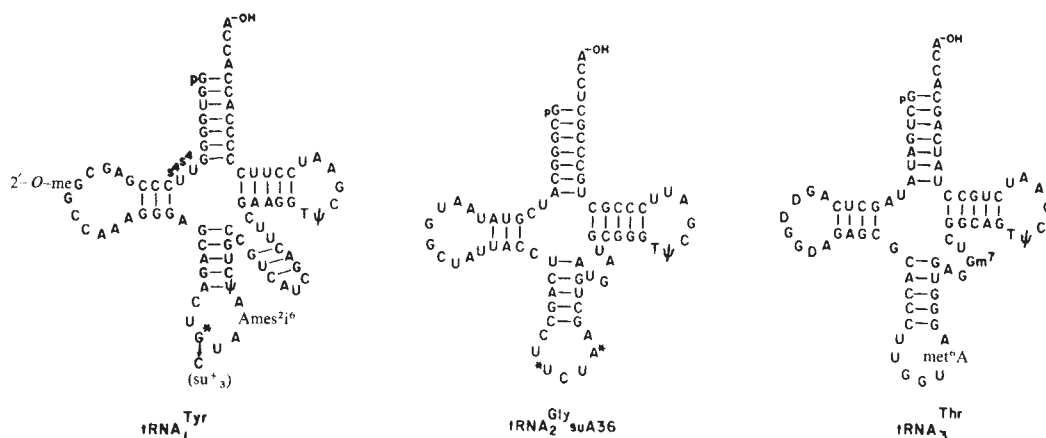


Fig. 1 Primary sequence of *E. coli* tRNA₁^{Tyr} (ref. 16), tRNA₂^{Gly} suA36 (ref. 10) and tRNA₃^{Thr} (ref. 9). s⁴U, 4-thiouridine; 2'-*O*-meG, 2'-*O*-methylguanosine; G*, modified guanosine¹⁷; mes³iA, 2-methylthio-6(Δ² isopentenyl) adenosine; ψ, pseudouridine; T, ribothymidine; D, 5,6-dihydrouridine; met⁸A, *N*-[9-(β-D-ribofuranosyl)purin-6-yl]-*N*-methyl-carbamoyl] threonine (non-methylated form is t⁶A); me⁷G, 7-methylguanosine; U*, unidentified derivative of uracil; A*, derivative of t⁶A.

Table 1 Levels of nucleotide modifications in *in vitro* synthesised tRNA

Modified nucleotide	Molar yield	
	Expected	Formed*
ψp	2	2
Tp	1	1
2'-OmeGp	1	—
G*p	1	—
s ⁴ Up	1†	0.74‡
mes ² i ⁶ Ap	1	0.62–0.75

	For tRNA ^{Thr} tRNA ^{Gly} (1:1 mixture)	
ψp	2	2
Tp	2	2
me ⁷ Gp	1	0.75
t ⁶ Ap	2	1.28§
Dp	1	—
U*p	1	—

*Molar yields of modified nucleotides determined by paper chromatography of T₂ RNase hydrolysates of the respective T₁ oligonucleotides extracted from fingerprints.

†In tRNA₁^{Tyr} synthesised *in vitro* in presence of α -³²P-UTP, only one of the two s⁴Up residues (U₉) is expected to be labelled by nearest-neighbour α -³²P-UMP distribution.

$^3\text{S}^4\text{U}$ was measured in $\text{tRNA}_{\text{P}^{\text{yr}}}$ only.

§ Molar yield of t⁶A was determined from two-dimensional thin-layer chromatography as described in Fig. 3, assuming two residues in the 1:1 mixture of tRNA^{Thr}/tRNA^{Gly}.

5% polyacrylamide-7 M urea gel, as described previously¹. tRNAs were located by autoradiography, extracted from the gel and digested by T₁ or T₂ RNase. Modified nucleosides, obtained as a result of the enzymatic activities present in S-100 extracts, were identified by two-dimensional thin-layer chromatography of the 3' mononucleotides from T₂ RNase hydrolysates. Alternatively, oligonucleotides were isolated from two-dimensional fingerprints of T₁ RNase digestion fragments, hydrolysed by alkali or T₂ RNase, and the modified nucleotides identified by paper chromatography⁷.

To study the formation of pseudouridine (ψ), the *in vitro* synthesised tRNAs were labelled by incorporation of α - ^{32}P -CMP. tRNA $_1^{\text{Tyr}}$ and tRNA $_2^{\text{Tyr}}$ contain two ψ residues: one in the T $_1$ fragment T ψ CG and a second near the anticodon in the oligonucleotides ACUCUAAA ψ CUG or UAAA ψ CUG (Fig. 1). tRNA $^{\text{Thr}}$ and tRNA $^{\text{Gly}}$ contain one ψ each in the T ψ CG fragments. Digestion of the α - ^{32}P -CMP-labelled oligonucleotides by alkali generated ^{32}P -labelled ψ MP which was then identified by paper chromatography (Fig. 2). Results show that incubation of the primary transcription products with dialysed *E. coli* S-100 extracts, a complete modification of U to ψ occurs in the T ψ CG fragments derived from tRNA $_1^{\text{Tyr}}$ (Fig. 2a, strip 2), tRNA $_2^{\text{Tyr}}$, tRNA $^{\text{Thr}}$ and tRNA $^{\text{Gly}}$ (Fig. 2b, strips 1 and 3). Formation of ψ in the anticodon region of tRNA $^{\text{Tyr}}$ was also quantitative

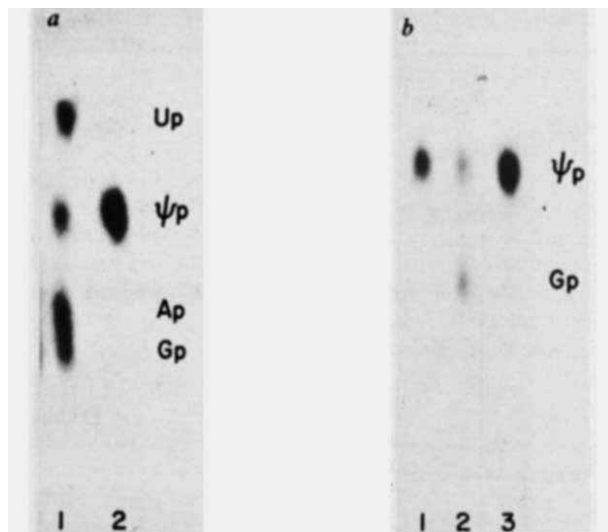


Fig. 2 Paper chromatography of hydrolysates of ψ p-containing T_1 oligonucleotides from *in vitro* synthesised tRNAs. $tRNA_1^{Tyr}$, $tRNA_2^{Tyr}$, $tRNA^{Gly}$ and $tRNA^{Thr}$ were synthesised *in vitro* by transcribing 20 μ g $\phi 80psu_3^{+/-}$ or λ h80T DNA in 200 μ l reaction mixtures containing 0.05 M Tris-HCl (pH 7.9), 0.01 M $MgCl_2$, 0.05 M KCl, 1 mM dithiothreitol, 0.4 mM each of ATP, GTP and UTP, 60 μ M α - ^{32}P -CTP (10.3 Ci mmol $^{-1}$; Amersham, England), 12 μ g ρ termination factor and 160 U RNA polymerase (1,600 U mg $^{-1}$). Synthesis was initiated after 5 min preincubation at 38 °C by addition of nucleoside triphosphates. After 30 min incubation at 38 °C the reaction was stopped by addition of actinomycin D (20 μ g) and DNase 1 (2 μ g). The mixture was incubated with 400 μ g dialysed S-100 extract for 90 min at 38 °C. Synthesised tRNAs were phenol extracted, purified by acrylamide gel electrophoresis, digested by T_1 RNase and the resulting oligonucleotides fractionated by two-dimensional electrophoresis as described previously¹. ψ p-containing oligonucleotides from fingerprints were extracted, alkali digested and analysed for nearest-neighbour distribution of α - ^{32}P -CMP by paper chromatography in an isopropanol-HCl-H $_2$ O (680 : 176 : 144 v/v) system⁷. **a**, Hydrolysates of (1) ACUCUAAA ψ CUG(C) and (2) T ψ CG derived from $tRNA_1^{Tyr}$ su_3^{+} ; **b**, hydrolysates of (1) T ψ CG, (2) UAAA ψ CUG(C) derived from $tRNA_2^{Tyr}$, and (3) T ψ CG from $tRNA^{Tyr}$ and $tRNA^{Gly}$. DNA-dependent RNA polymerase was prepared from *E. coli* MRE-600 cells²⁰ and further purified by low salt glycerol centrifugation²¹. Termination factor ρ (ref. 14) and S-100 extract¹ were prepared from *E. coli* MRE-600.

and the nearest-neighbour distribution of CMP was as expected —1 (ψ p):1 (Up):1 (Ap):1 (Gp) for ACUCUAAA ψ CUG(C) of $tRNA_1^{Tyr}$ su_3^{+} and 1(ψ p):1(Gp) for UAAA ψ CUG(C) of $tRNA_2^{Tyr}$ (Fig. 2a, strip 1 and Fig. 2b, strip 2).

From these results it may be concluded that the *in vitro* synthesised tRNA can serve as a substrate for the two enzymatic activities⁸ responsible for the formation of ψ in the T ψ CG and in the anticodon regions of the tRNA. Formation of ψ is the only modification observed in the *in vitro* synthesised tRNAs as a result of incubation with dialysed S-100 extracts. To study the methylations leading to the formation of T and me⁷G or the thiolation leading to the formation of s⁴U, tRNA molecules were synthesised *in vitro* with α - ^{32}P -UTP and incubated with dialysed S-100 extract in the presence of S-adenosyl-L-methionine or L-cysteine, respectively. Figure 3a shows the two-dimensional thin-layer chromatography of a T_2 RNase digest of $tRNA^{Thr}$, $tRNA^{Gly}$ (1:1 tRNA mixture migrating as a single band on acrylamide gel¹) in which the methylated nucleotides Tp and me⁷Gp can be identified. Tp was also determined in the T ψ CG fragments isolated from two-dimensional fingerprints of T_1 RNase digests. Yield of Tp was found to increase with S-adenosyl-L-methionine concentration, reaching 100% at 1.15×10^{-4} M. Formation of me⁷G was inferred also from the presence of the oligonucleotide me⁷GUCG in a two-dimensional fingerprint of the T_1 digest [of α - ^{32}P -UMP-labelled $tRNA^{Thr}$ (data not shown). me⁷G was identified by paper chromatography of a T_2 digest of the oligonucleotide, and an efficiency of 75% measured for me⁷G synthesis.

Figure 3b shows the modification of U₉ of $tRNA_1^{Tyr}$ to s⁴U (yield 74%). The synthesis of the hypermodified bases mes²i⁶A (in $tRNA_1^{Tyr}$ and $tRNA_2^{Tyr}$) and t⁶A (in $tRNA^{Thr}$, $tRNA^{Gly}$) is illustrated in Fig. 3c and d. tRNAs were synthesised with α - ^{32}P -ATP and incubated with S-100 extracts in the presence of specific cofactors as described in Fig. 3c and d. A yield of 62–75% was observed for mes²i⁶A and 64% for t⁶A modifications of the specific A residues. Note, however, that A₃₇ in $tRNA^{Thr}$ was modified only to t⁶A and was not further methylated to met⁶A as expected from the studies of Clarke and Carbon⁹. In addition it was observed that the molar yield of t⁶A in the *in vitro* synthesised $tRNA^{Thr}$ $tRNA^{Gly}$ mixture exceeded the expected value for a single t⁶A residue present in $tRNA^{Thr}$, thus supporting data of Roberts and Carbon¹⁰ showing the presence of a t⁶A derivative at A₃₇ in $tRNA^{Gly}$ su^{+} A36. The molar yields of the nucleotide modifications obtained in *in vitro* synthesised tRNA molecules are summarised in Table 1.

To test the amino acid accepting ability of the *in vitro* synthesised tRNA molecules, $\phi 80psu_3^{+/-}$ and λ h80T DNAs were transcribed by purified RNA polymerase in the presence or absence of termination factor ρ and the synthesised tRNA precursors were processed by a tRNA-free dialysed *E. coli* extract. The resulting tRNAs were extracted with phenol, precipitated using ethanol and assayed for acceptance of ³H-tyrosine, ³H-threonine and ³H-glycine. Figure 4 shows the linearity of aminoacyl-tRNA formation as a function of *in vitro* synthesised tRNA. Note the enhancing effect of ρ , when present

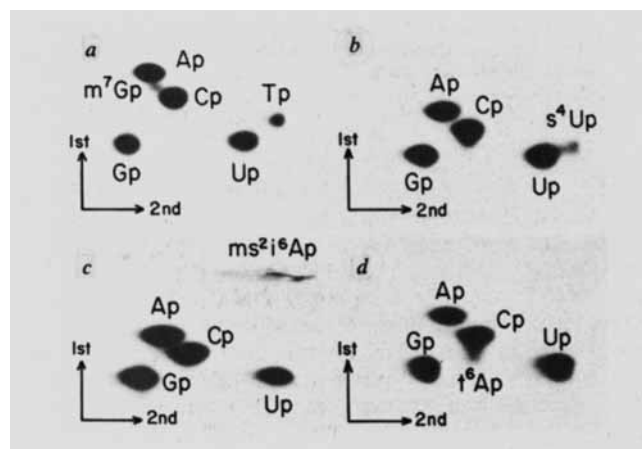


Fig. 3 Autoradiography of two-dimensional thin-layer chromatography of T_2 digests of *in vitro* synthesised and modified tRNAs. **a**, α - ^{32}P -UMP-labelled $tRNA^{Thr}$ $tRNA^{Gly}$ mixture (1:1) was synthesised *in vitro* by transcription of λ h80T DNA, followed by 90-min incubation with 2.2 mg ml $^{-1}$ dialysed *E. coli* S-100 extract, 0.12 mM S-adenosyl-L-methionine and 0.6 mM EDTA. **b**, α - ^{32}P -UMP-labelled $tRNA_1^{Tyr}$ was synthesised by transcription of $\phi 80psu_3^{+/-}$ DNA followed by 90-min incubation with 2.2 mg ml $^{-1}$ dialysed *E. coli* S-100 extract, 1 mM L-cysteine, 5 mM ATP, 0.05 mM pyridoxal phosphate and 10 mM β -mercaptoethanol. α - ^{32}P -ATP-labelled $tRNA_2^{Tyr}$ (**c**) and α - ^{32}P -ATP-labelled $tRNA^{Thr}$ / $tRNA^{Gly}$ (**d**) were synthesised by transcription of λ h80T DNA followed by 90 min incubation with 4.4 mg ml $^{-1}$ non-dialysed *E. coli* S-100 extract, (0.2 mM) Δ^3 -isopentenyl pyrophosphate (Fox chemicals), 4 mM ATP, 1 mM L-cysteine, 0.12 mM S-adenosyl-L-methionine, 10 mM β -mercaptoethanol, 450 μ g ml $^{-1}$ isopentenylpyrophosphate isomerase¹⁸, 0.4 mM L-threonine and 25 μ g ml $^{-1}$ folic acid. tRNAs were extracted with phenol, precipitated using ethanol, separated by acrylamide gel electrophoresis and extracted as described previously¹. T_2 RNase hydrolysis was as described by Barrell⁷ and the two-dimensional chromatography followed the method of Nishimura¹⁹. Molar yields of modified nucleotides were determined by summing radioactivity found in all the spots from the respective plate. This number was normalised to the expected number of moles of nucleotides in a nearest-neighbour position to the incorporated α - ^{32}P -UMP, -AMP or -CMP as determined from the primary sequence of the respective tRNA (Fig. 1). The fraction of radioactivity in the modified nucleotide spot related to the calculated radioactivity for a single nucleotide gives the molar yield of modification.

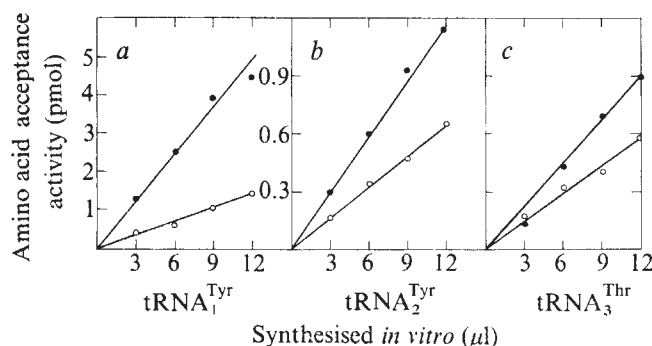


Fig 4. Amino acid acceptance of *in vitro* synthesised tRNAs. tRNA₁^{Tyr} (a), tRNA₂^{Tyr} (b) and tRNA₃^{Thr} (c) were synthesised *in vitro* in the presence (●) or absence (○) of 12 μg p factor in 200 μl reaction mixtures containing 0.05 M Tris-HCl (pH 7.9), 0.01 M MgCl₂, 0.05 M KCl, 1 mM dithiothreitol, 0.4 mM each of ATP, CTP, UTP and GTP, 20 μg φ80psu₃⁺ DNA or 30 μg λh80T DNA, and 160 U RNA polymerase (1,600 U mg⁻¹). Synthesis was initiated after 5 min preincubation at 38 °C by addition of nucleoside triphosphates. After 30 min incubation at 38 °C the reaction was stopped by addition of 20 μg actinomycin D and 2 μg DNase I. tRNA-free S-100 extract (320 μg) was added and the incubation continued for 90 min. Synthesised tRNA was extracted with phenol, precipitated with ethanol and dissolved in 45 μl water. Aliquots of 3, 6, 9 and 12 μl were assayed for amino acid acceptance in 50 μl reaction mixtures containing 0.04 M Tris-HCl (pH 7.5), 0.01 M MgCl₂, 2 mM ATP, 3 mM glutathione, 1.5 μM L-3,5-³H-tyrosine (41 Ci mmol⁻¹, Amersham UK) or 11 μM L-3,5-³H-threonine (1.8 Ci mmol⁻¹, New England Nuclear), 19 unlabelled amino acids (except tyrosine or except threonine respectively) and purified tyrosyl-tRNA synthetase²² or 16 μg tRNA-free S-100 extract containing threonyl-tRNA synthetase. Incorporation of ³H-labelled amino acids into acid-insoluble material was measured after 10 min incubation at 37 °C. tRNA-free S-100 extract was prepared by passing an *E. coli* S-100 extract adjusted to 0.4 M KCl through a DEAE-cellulose column.

during tRNA gene transcription on the amounts of amino acid-accepting tRNAs produced. Figure 4 shows that the presence of p increases the amount of tRNA₁^{Tyr} transcribed on φ80psu₃⁺ DNA by a factor of 3–4 and that of the tRNA₂^{Tyr} and tRNA₃^{Thr} transcribed on λh80T DNA by a factor of 1.5–2. These results confirm our previous observations concerning the effect of p factor on tRNA synthesis^{11,12}. We have shown¹³ that the presence of p during transcription of the tRNA₁^{Tyr} gene increases the amounts of tRNA precursor produced without affecting its size. Thus, although it is generally assumed that p factor affects only the termination of an RNA molecule¹⁴ our results do not indicate a termination effect for p in tRNA gene transcription. Note that transcription of tRNA genes by purified RNA polymerase also takes place in the absence of p and does not require any additional factor as suggested by Bikoff and Gefter^{5,6}.

The efficiency of production of amino acid chargeable tRNA molecules in the *in vitro* system is remarkable. From the data reported in the legend to Fig. 4 it can be calculated that the transcription of approximately 1.4 pmol tRNA₁^{Tyr} gene DNA (20 μg φ80psu₃⁺ DNA of molecular weight 30 × 10⁶, containing 2 tRNA₁^{Tyr} genes) resulted in the formation of 5.6 and 19.5 pmol tRNA₁^{Tyr} when synthesised in the absence or presence of p respectively. Transcription *in vitro* of λh80T DNA was found to produce tRNA₂^{Tyr} and tRNA₃^{Thr} in equivalent amounts as determined by their amino acid acceptor activities (Fig. 4b and c). The glycine-accepting capacity of synthesised tRNA₁^{Gly} was only 50% of that measured for tRNA₂^{Tyr} or tRNA₃^{Thr} (data not shown). This is probably due to the lower affinity of glycyl-tRNA synthetase for the tRNA₂^{Gly}su+A36 species¹⁵.

From the present *in vitro* studies we conclude that purified RNA polymerase alone can efficiently transcribe tRNA genes to produce primary tRNA precursors. The high fidelity of the transcription is illustrated by the fact that the synthesised RNA molecules are recognised as substrates by processing nucleases, by nucleoside modifying enzymes and by the aminoacyl-tRNA

synthetases. Note that tRNAs synthesised *in vitro*, when processed by dialysed S-100 extracts, are (with the exception of ψ formation) completely unmodified. The unmodified tRNA precursors can be correctly processed and the resulting unmodified tRNAs are capable of charging with the corresponding amino acid. These findings argue against a possible role of the modified bases in tRNA precursor processing or tRNA aminoacylation. The present study shows that the *in vitro* synthesised tRNA is a suitable substrate for the study of the enzymes involved in nucleoside modifications and may serve to elucidate further the role of the modified nucleosides in the biological functions of mature tRNA.

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Evolutionary origin of 5.8S ribosomal RNA

IN prokaryotes, the genes for 5S, 16S and 23S ribosomal RNA are linked in a polycistronic transcriptional unit^{1,2}. In eukaryotes, on the other hand, the analogous unit contains the genes for 5.8S, 18S and 28S rRNAs, whereas the 5S RNA genes are found elsewhere in the genome. The analogous positions occupied by prokaryotic 5S RNA genes and eukaryotic 5.8S RNA in their respective genomes prompted Doolittle and Pace to speculate that these two RNA classes, both of which are components of the large ribosomal subunit, might have a common evolutionary origin³. If these RNAs are in fact homologous, one would expect their sequences to bear some relationship to each other. We report here our evaluation of possible structural relationships between known sequences of 5.8S and 5S rRNA, and conclude that they have no common evolutionary origin.

The comparison of RNA sequences is extremely difficult when insertions and deletions have to be considered. Recently, however, we have developed a test of similarity which can be applied to RNA sequences and which involves the establishment of the best alignment (most identities) of two sequences allowing a fixed number of insertions/deletions⁴. The number of positions in the alignment which are occupied by identical bases in the two sequences is counted. The process is repeated, increasing the allowed number of insertions/deletions by one. The number of identities are then compared with like values computed

Table 1 Comparison of 5S and 5.8S RNA sequences

Experiment	RNA	Insertions or deletions	Identities	Identities in random sequences
1	5.8S yeast	2	112	61
	5.8S hepatoma			
2	5.8S yeast	8	67	66
	5S <i>E. coli</i>			
3	5.8S yeast	8	66	65
	5S KB cells			
4	5.8S yeast	8	62	60
	5S protopkaryote			
5	5.8S yeast	8	58	61
	5S protoeukaryote			
6	5.8S hepatoma	8	67	69
	5S <i>E. coli</i>			
7	5.8S hepatoma	8	63	66
	5S KB cells			
8	5.8S hepatoma	8	61	61
	5S protopkaryote			
9	5.8S hepatoma	8	59	64
	5S protoeukaryote			

*The number of identities between random sequences is an average of five different experiments. Random sequences have the same composition and length as the two real sequences being compared in each experiment. We have chosen to give the number of identities found permitting eight insertions or deletions, since these are representative of values obtained permitting either more or less.

from random sequences. Related sequences show statistically significant increases in the number of identities found for each increase in the number of insertions/deletions permitted, up to a certain limit.

Previously, we have shown that 5S RNAs of eukaryotes and prokaryotes are homologous using this test. When applied to the comparison of the two known sequences of 5.8S RNA^{5,6}, an unequivocal relationship is apparent (see Table 1). It would seem, given the relatively large difference between the 5S sequences from yeast and humans^{7,8}, that 5.8S RNAs are more conservative to evolutionary change than eukaryotic 5S RNA. Such resistance to the fixation of natural mutations usually indicates an important structural and functional role. In fact, the sequences of all mammalian 5S RNA are apparently identical⁹ and the same holds for 5.8S RNA⁶. The fact that both molecules are evolutionarily conservative increases the probability of finding a common origin, if in fact one exists.

As shown in Table 1, the test when applied to the comparison of either the eukaryotic or prokaryotic 5S RNA sequence with either 5.8S RNA sequence (yeast and hepatoma) shows them to be no more related than random sequences.

One might argue that in spite of the conservative evolution of these molecules, all similarities of sequences have been wiped out during their independent evolution from a hypothetical ancestor. We have, however, developed methods to infer hypothetical ancestral sequences using known 5S RNA sequences¹⁰. With a corpus of 15 known sequences we have estimated ancestral prokaryote and eukaryote sequences: no 5S RNA ancestral sequences show any relationship to known 5.8S RNA sequences.

These results indicate that 5.8S RNA does not share a common ancestor with 5S RNA and therefore did not arise by duplication of the 5S RNA gene followed by divergence. One interesting possibility is that the 5.8S RNA evolved from the intergenic region of the bacterial ribosomal genome.

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Bean halo-blight toxin

Pseudomonas phaseolicola causes a disease of beans commonly called halo blight. The halo is a chlorotic zone which appears in the leaf round each infection site. It has been interpreted as the effect of a toxin produced at, and diffusing away from, the infection site. The zone characteristically contains a relatively large amount of the amino acid ornithine¹. When *Ps. phaseolicola* was grown in a defined liquid culture medium at 18 °C the filter-sterilised medium, when applied to bean leaves, caused the chlorosis symptom of the disease². From such media I have isolated a substance, 80-90% pure, which, when introduced to the leaf at a level of 100 ng per g fresh weight, causes both chlorosis and accumulation of ornithine. I report here the highly unusual structure of this compound. Its extreme lability in the presence of weak acids has made its isolation difficult, but has helped in the resolution of its structure.

The pure substance is water soluble, and hydrolysis with 6 M HCl (100 °C, 18 h) liberates three amino acids (ornithine, alanine and homoarginine). In spite of the presence of two basic amino acids (ornithine and homoarginine) the toxin itself behaves during electrophoresis at pH 2 like the acidic amino acids, suggesting that it also contains strong acid groups. More gentle hydrolysis of the toxin with dilute HCl (0.01 M, 40 °C, 2 h) yields a new compound which contains the same amino acids as the toxin but which shows no toxic properties. This new compound has, at pH 2, an electrophoretic mobility a little greater than that of ornithine, and now consistent with its content of basic amino acids. Sequential cleavage with carboxypeptidase and gas chromatography-mass spectrometry showed it to be the tripeptide ornithylalanylhomarginine (structure 1, Fig. 1).

Because ornithine is the N-terminal amino acid of the tripeptide, both ornithine amino groups are unbonded. When, however, the intact toxin was reacted with 1-fluoro-2,4-dinitrobenzene and the product was hydrolysed, N^α-dinitrophenylornithine was the only amino acid derivative produced. The tripeptide, in contrast, gave N^α,N^δ-di-dinitrophenyl-ornithine. This shows that the δ-NH₂ of the toxin is bonded. There are two possible interpretations: either the toxin structure is cyclic (for example, cyclic-N^δ-ornithylalanylhomarginyl) incorporating the ornithyl side chain in the ring (in which case dilute acid must open the ring at the ornithyl-N^δ-peptide bond), or the toxin structure is linear with a non-peptide group bonded to the ornithyl-N^δ (in which case dilute acid would yield peptide and non-peptide products; structure 2, Fig. 1).

The former was excluded when the action of carboxypeptidase enzymes on the toxin was studied. Homoarginine, then alanine, were sequentially cleaved, yielding a product which contained only one amino acid, ornithine. Because carboxypeptidase requires the presence of a free terminal carboxylic acid group to be able to act on the adjacent peptide bond (hence its sequential action), the cyclic structure would not have been attacked by it. I therefore conclude that the toxin is represented by structure 2, and that there is a further group present that is liberated from the toxin by the action of dilute acid.

matters arising

Niche breadth in Bryozoans

O'CONNOR *et al.*¹ consider a resource gradient with an optimum point for a particular species. Other factors being equal, individuals migrating to a new environment will tend to select the optimum, or near optimum. As population density increases, the advantages of being near the optimum may be offset by intraspecific competition². Migrants may therefore choose a sub-optimal point on the resource gradient. As population density increases so will the range of resource gradient used.

O'Connor *et al.*¹ use the phenomenon of an increase in range with population density as a test for intraspecific competition. This is not a suitable test because range will increase with population density even in the absence of intraspecific competition. If intraspecific competition was not occurring and individuals chose the resource optimum with an associated variance from a normal distribution (say, mean of zero and variance of unity), then the expected range would increase with population density (going from five to fifty individuals the expected range would increase from 2.32 to 4.50 (ref. 3)). It is not necessary to postulate a normal distribution or even a resource optimum for an expected increase of range with population density.

Using the epiphytic bryozoan *Alcyonidium hirsutum* settling on the serrated wrack *Fucus serratus*, it is probably permissible to replace the percentage cover used by O'Connor *et al.*¹ by the numbers of individuals over a wide range of settlement density, provided that settled individuals are of the same age⁴. The optimum on the particular resource gradient used has been investigated previously⁵.

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⁴ Hayward, P. J., and Harvey, P. H., *J. mar. biol. Ass. U.K.*, **54**, 677–684 (1974).

⁵ Hayward, P. J., and Harvey, P. H., *J. mar. biol. Ass. U.K.*, **54**, 665–676 (1974).

O'CONNOR ET AL. REPLY—We accept the point raised by Harvey *et al.*¹ but it does not alter our earlier conclusion that *Alcyonidium hirsutum* shows increased niche breadth in the presence of intraspecific competition and decreased niche breadth in the presence of interspecific competition. Range on the resource gradient is indeed correlated statistically with the number of bryozoan settlements on each *Fucus serratus* frond, but because these fronds vary in age along their length, it is less reasonable to assume that the resulting colonies should be of equal age. In practice the partial correlation coefficient between *Alcyonidium* abundance (percentage cover) and its range, controlling for the number of faces colonised on each plant (and thus for the effect suggested by Harvey *et al.*) was 0.506 ($P < 0.05$). That is, *Alcyonidium* shows increased range in resource utilisation as its own population levels rise, and does so independently of the statistical artefact suggested.

For the other three bryozoan species whose distribution we analysed, we reported small positive correlations between range and abundance, in small samples containing some unmeasured interspecific competition. Correcting for the possible statistical effect by partial correlation left us with positive correlations in the case of *Flustrellidra hispida* ($r_p = 0.419$, $P < 0.2$) and *Electra pilosa* ($r_p = 0.215$, $P < 0.3$), but with a negative value in the case of *Membranipora membranacea* ($r_p = -0.463$, $P < 0.2$). Thus only for this species might our first results have originated in a sample-size effect, though as we pointed out previously, we have available to us only samples which are subject to some degree of interspecific competition which tends to reduce niche breadth anyway. In summary, therefore our results still indicate that intraspecific and interspecific competition lead respectively to increase and decrease in niche breadth in *A. hirsutum* and possibly in other bryozoan species as well.

We thank Alec Vardy for reviewing our statistical argument.

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¹ Harvey, P. H., Ryland, J. S., and Hayward, P. J., *Nature*, **260**, 77 (1976).

THE claim of O'Connor *et al.*¹ that distributional and density data for the bryozoan *Alcyonidium hirsutum* growing on *Fucus* fronds demonstrate the effects of both intra- and interspecific competition on niche width is unsound for both methodological and theoretical reasons. A positive correlation would be expected between the number of bryozoan colonies and the 'population level' as measured by percentage cover, and therefore the positive relationship found between the range of segments utilised and the population level is explicable without recourse to competition theory. On fronds which are more suitable overall, some of the less favoured segments would become acceptable, so that the frond is colonised more heavily and over a wider range of segments. Other models will also produce this result. If darts are thrown at a linear target the range between darts should increase with the dart 'population size'.

Regarding interspecific competition we note that in the no-, one-, and two-competitor cases, decreasing sample sizes are used. A narrower niche width would thus be expected in the cases with competitors irrespective of interspecific effects. The utilisation curves for the first two cases, however, do not differ significantly (Kolmogorov–Smirnov two-sample test²), negating conclusions based on supposed differences between them. The two-competitor curve is based on too few colonised segments (18) for much confidence to be placed in supposed trends; furthermore, when allowance is made for the modal shift there is no significant difference between this and the non-competitor curve. Finally, B.

$$(1nB = -\sum p_i \ln p_i)$$

is an accepted measure of niche width^{3,4}. The value for the one-competitor case is higher than for the no-competitor curve, contrary to the prediction of O'Connor *et al.*¹.

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² Pianka, E. R., *Evolutionary Ecology* (Harper and Row, New York, 1974).

³ Fisher, R. A., and Yates, F., *Statistical Tables for Biological, Agricultural and Medical Research* (Oliver and Boyd, London, 1963).

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O'CONNOR ET AL. REPLY—Crozier and Day¹ offer four distinct criticisms of our analysis² of niche breadth in Bryozoa. They suggest, first, that on fronds which are more suitable overall some of the less favoured segments would become acceptable, yielding a competition-free correlation between range and population level. As we pointed out explicitly in our paper, however, the available space at the optimum height is not physically filled at the higher population levels, so that animals settling elsewhere are behaving maladaptively if intraspecific competition is absent. Competition theory readily explains such suboptimal settlement in terms of a balance between the disadvantages of being away from the competition-free optimum of the resource gradient and the advantages of the reduced intraspecific competition found away from the optimum^{3,4}; the model suggested by Crozier and Day¹ leaves us, on the other hand, with unexplained maladaptive behaviour on the part of the bryozoans.

The linear dart target criticism advanced is essentially that of Harvey *et al.*⁵, to which we have responded with a partial correlation analysis⁶ demonstrating that increase in resource range with increasing *Alcyonidium* population size was independent of the number of observations or 'darts'. We note in addition here that the dart effect is in any case too small to account for our results. Thus, if the change in *Alcyonidium* numbers between 2% and 4% cover (the latter being our median value) involved an increase of as few as 2 individuals at settlement, the dart effect predicts⁷ only an increase in range of 84%, whereas we empirically observed an increase of 178%; for a slightly more realistic change of from 10 to 20 individuals, the dart effect is even less (21.4%), increasing the shortfall from the observed change even further. Similar calculations over other ranges of population size confirm our original conclusion, since the observed range increases are always substantially larger than any possible effect of sample size.

The third criticism by Crozier and Day¹, that the effect of sample size could explain our interspecific results, is incorrect on two grounds. First, for our interspecific analysis we used not the range of the resource utilisation function but rather its width at half height. This measure more closely resembles a standard deviation than a range in its statistical behaviour, that

is, it is largely insensitive to changes in sample size. Second, even if this measure had been subject to the effect of sample size, the resulting increase would have been too small to account for the observed changes in niche breadth. Thus, for *Alcyonidium* our sample size effectively doubled (from 69 to 149) between the one-competitor and no-competitor situations: Fisher and Yates' table⁷ shows that the effect of such a doubling of sample size would have been less than 14%, whereas the observed increase was of 34%, substantially larger. Similar calculations for the other seven comparisons show that in six cases the dart effect is too small by even bigger factors to account for the observed increases: only for the change in *Membranipora* niche breadth between the one- and two-competitor situations could an effect of sample size have accounted for the observed change. Therefore, even after assuming we had used range, and not the more stable width at half height, as our measure of niche breadth, the effects of the differences in sample sizes are so small that after making full allowance for them, seven of the eight comparisons are still indicative of competitive effects (Sign test⁸, $P < 0.05$), that is, the data still demonstrate that the presence of interspecific competitors on fronds reduces bryozoan niche breadths on those fronds. We thus disregard this criticism of Crozier and Day¹.

Their final criticism is that the analyses chosen by them failed to support our conclusions. We note first that they overstate their case with respect to the Kolmogorov-Smirnov test: Siegel⁸ explicitly states (p. 129) that the failure of this test to reject a null hypothesis (here that the one-competitor and no-competitor curves do not differ) does not mean that the hypothesis should then be accepted, contrary to the assertion of Crozier and Day. Second, we note that the formula

$$\ln B = -\sum p_i \ln p_i$$

requires that niche breadth be related to the Shannon-Wiener index of species diversity, H , by $B = \exp(H)$. Niche breadth and species diversity are thus mathematically related. Since we know from empirical evidence⁹ that different diversity indices lead to different biological conclusions, even when applied to the same data, we are unable to accept any one of the related niche breadth formulae as definitive. The widespread acceptance of any particular formula may merely reflect uncritical acceptance of its hidden assumptions. The measure of niche breadth we used has the merit of being distribution free and has an even more widespread acceptance in mea-

suring distribution widths¹⁰ than the information theory measure tested by Crozier and Day. We would naturally like to see all niche breadth measures tested with our data supporting our conclusions, but when simple measures support biologically sensible conclusions and complex measures fail to do so, we feel it appropriate to suggest that maybe the mathematics, rather than the biology, is at fault.

In summary, therefore, the criticisms offered by Crozier and Day¹ are only partially valid, and have too small an effect to invalidate our conclusions.

We thank Jim Gilliam for helpful discussion.

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Enteropancreatic circulation of digestive enzyme

WE are studying highly specific intracellular proteinases and have read with interest yet alarm the two recent reports^{1,2} by Rothman *et al.* proposing an enteropancreatic circulation for digestive enzymes. This seems unlikely for the following reasons (accepting the premise that protein molecules can be transported across intestinal and pancreatic acinar cells).

If the proteins are absorbed into the bloodstream in the active enzyme forms (chymotrypsin and trypsin, for example) they would be inactivated very rapidly by the plasma inhibitors such as α_1 -antitrypsin, α_1 -antichymotrypsin and α_2 -macroglobulin before they could hydrolyse physiological substrates like prothrombin, fibrinogen, plasminogen and kininogen. If any active molecules were lucky enough to escape this bombardment by circulating inhibitors and be reabsorbed by the pancreas, what a cruel fate to be inactivated at the last hurdle by the pancreatic inhibitors.

If the proteins are absorbed in the non-activated forms of chymotrypsinogen and trypsinogen, they might just escape the attention of the inhibitors and be reabsorbed by the pancreas

but to what avail? If an excess of "protein" is secreted into the intestine, Rothman *et al.* seem to be proposing that a large percentage of this can be conserved by recycling. The activation processes for zymogens such as chymotrypsinogen and trypsinogen would, however, seem to be too rapid and complete to allow any significant level of zymogen to remain. The digestive capacity is controlled, not by the degree of zymogen activation in the intestine, but by the absolute amount of zymogens released on hormonal stimulus of the pancreas.

Space will not permit critical evaluation or alternative explanation for Rothman *et al.*'s observations. Their experiments have been conducted by following "insensitive" parameters such as protein output by the pancreas and chasing a few radioactive counts around the bloodstream. It would be illuminating to see the experiment done wherein (potential) enzymic activity is followed. Only then would we have to consider the feasibility of an enteropancreatic circulation.

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¹ Götze, H., and Rothman, S. S., *Nature*, 257, 607 (1975).

² Liebow, C., and Rothman, S. S., *Science*, 189, 472 (1975).

ROTHMAN, GÖTZE AND LIEBOW REPLY—We are sorry that our observations and ideas have alarmed Kay and Beynon¹; however, new scientific findings, are appropriately met with interest, not fear. They feel that our observations must be explained in other, although unspecified, ways because in their view the circulation of digestive enzyme is quite impossible. If we understand their position correctly, they argue that all active proteolytic enzyme would be irreversibly bound in blood and that proenzyme entering the intestine would be immediately and completely activated in all circumstances and thus be unavailable for circulation. Since these postulates refer only to proteolytic enzyme and since a large percentage of the pancreatic digestive enzymes are not proteolytic, Kay and Beynon are apparently not questioning the existence of a circulation of digestive enzyme, but more narrowly the circulation of proteolytic digestive enzymes. We have directed our response to this question.

Although the workings and physiological functions of plasma protein inhibitors are not completely understood, certainly the mere presence of various inhibitors in the blood (or acinar cell) does not mean that all

Matters arising

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enzyme is irreversibly bound by these molecules and taken out of circulation. On the contrary, it is the presence of these inhibitors in blood that makes it reasonable to conceive of active proteolytic enzyme being circulated. The point is that to prove whether or not active proteolytic digestive enzyme is circulated is a matter for further investigation, not presupposition. Relative to the potential for proenzyme circulation, Kay and Beynon do not cite the evidence on which they base their contention—that the activation of enzyme in the intestine is "too rapid and complete to allow any significant level of zymogen to remain." We are unaware of such evidence. They should remember that maximal activation does not mean that all the enzyme molecules have been activated, and that the activation kinetics of a few defined components in a test tube does not necessarily reflect what goes on in the intestine in various physiological situations. Kay and Beynon apparently insist on restricting the potential meaning of a biological phenomenon to their view of present knowledge of the chemistry and function of certain molecules. They thereby assume that they understand the chemistry and function of these molecules fully and that there are no unknown elements in the piece. On what basis do they make these assumptions?

Finally, in spite of their preconceived notions, the enteropancreatic circulation of digestive enzyme is a fact documented, not by "chasing a few counts around the bloodstream" as they egregiously suggest, but by demonstrating, with tracer kinetics, that labelled chymotrypsinogen instilled in the intestine reappears in pancreatic secretion collected directly from the duct and exclusively in the chymotrypsinogen band of secreted proteins separated by electrophoresis.

In the recent paper² which prompted Kay and Beynon's letter¹, we described experiments which suggest that this circulation may account for the move-

ment of substantial amounts of protein. Two corollary observations further reinforce the interpretation that enzyme circulation is involved: (1) output (the increase in protein secretion) correlates strongly with input (digestive enzyme added to the intestinal lumen or injected into the bloodstream) with a slope of 0.92, or in other words, enhanced protein secretion by the pancreas accounted for 92% of protein input; and (2) these two variables fit the same regression line whether the collected secretion was injected into the bloodstream or instilled into the intestine. While these observations are subject to other interpretations (which were carefully pointed out in our article and which Kay and Beynon do not acknowledge in their letter), the circulation of substantial amounts of digestive enzyme is the most direct, and thus, the best interpretation of the results. It is on this basis that we feel it should be the hypothesis for the moment. If Kay and Beynon really have a more convincing interpretation of these experiments, then why do they hesitate to present it? We would greet it with interest, not alarm.

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² Götze, H., and Rothman, S. S., *Nature*, 257, 607 (1975).

Doubts about the role of the locus coeruleus in learning and the phosphorylation mechanism engaged in the cerebellum

GILBERT¹ has proposed a model of how the cerebellum, with the assistance of the locus coeruleus, could memorise movements. We have been studying the anatomy and chemistry of these and related systems, and have evidence that bears directly on that hypothesis.

The first point concerns the crucial role of the locus coeruleus in the memory consolidation of "motor signals stored in the cerebellum"¹. The evidence to support that view comes from one study² which observed the effects of locus coeruleus lesions on learning. The results have, however, been brought into question³. Using a different approach, our laboratory has been studying the effects of localised brain stimulation on learning and retention performances (see ref. 4). Quite remarkable localisations have been obtained in the medial nucleus of the amygdala⁵, substantia nigra, pars compacta⁶, medial and sulcal portions

of frontal cortex⁷ and the lateral portions of the medial forebrain bundle⁸. In these specific regions, but not in surrounding locations, brain stimulation during learning disrupted retention. Using the same paradigm we have found no effect of locus coeruleus stimulation⁷. The results cast doubts on the essentiality of the locus coeruleus to the learning and memory of movements.

Related to the issue of the involvement of the locus coeruleus in learning is the point made by Gilbert¹ that "the locus coeruleus... will support intracranial self-stimulation". Though it is not to be denied that intracranial self-stimulation (ICSS) can be obtained when electrodes are in the region of the locus coeruleus⁹, it is by no means proven that the locus coeruleus or its axonal system is responsible for that behaviour. Our evidence argues against a role for the locus coeruleus in intracranial self-stimulation. Intracranial self-stimulation in the region of the dorsal bundle, the efferent ascending pathway of the locus coeruleus, is unaffected by the bilateral destruction of the locus coeruleus¹⁰; and intracranial self-stimulation has not always been obtained when electrodes are in the locus coeruleus^{11,12}. Given the uncertainty of the locus coeruleus involvement in brain stimulation reward it seems premature to invoke a role for it in memorising learned movements.

The second point concerns the mechanism of storage proposed by Gilbert¹: the phosphorylation of membrane protein. We have studied the endogenous phosphorylation of four protein components of brain membranes¹³, and have found that one component, which we term band F, is altered by a training experience¹⁴. This effect is most noticeable in the caudate nucleus, but alterations (admittedly of a less dramatic nature) have also been observed in the cerebral cortex. An analysis of the vermis of the cerebellum, however, has yielded negative results; that is, there is no change in cerebellar membrane phosphorylation as a consequence of training such as was observed with membranes of the caudate nucleus. Although this represents an initial finding in two replicated experiments, the results gathered to date lend no support to Gilbert's views¹ on the location of the mechanism of storage. Actually, the phosphorylation mechanism itself may be appropriate, but its location may be elsewhere, for example, in the caudate nucleus, rather than in the cerebellum.

Though I am in sympathy with the approach offered by Gilbert, I question the detailed mechanism proposed, both with respect to the involvement of the locus coeruleus in learning and

reinforcement and with respect to alterations in phosphorylation within the cerebellum. Our data, though negative on both specific points, point to particular brain locations potentially involved in memory, and to related regions where chemical storage mechanisms could be engaged following a learning experience.

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DR GILBERT REPLIES—To support my proposal about motor learning in the cerebellum¹, I cited a study² which showed that locus coeruleus lesions markedly impaired, or abolished, the ability to learn to run with increased speed in an L-shaped runway for a food reward. Routtenberg questions the result of my study (see refs 1 and 2) because there seems to be no learning deficit in a T-maze discrimination task for animals with locus coeruleus lesions³. The types of learning being tested in those studies are, however, quite different, and the results are not necessarily contradictory. I have described^{1,4} how the cerebellum could store information required for the production of coordinated muscular activity in a learned movement. I have not suggested that the cerebellum is involved in other types of learning such as T-maze discrimination, and the fact that there is no learning deficit for this task with locus coeruleus lesions is irrelevant to my proposal.

Stimulation of the locus coeruleus probably leads to an increased release of noradrenaline at Purkyne cells⁵ and could therefore lead to enhanced retention. The enhancement of retention by central administration of noradrenaline only occurs after depletion of central noradrenaline stores⁶, however, and the

stimulation of the locus coeruleus could be expected to enhance retention only in similar conditions. Therefore, the negative results of locus coeruleus stimulation⁷ do not conflict with its proposed role¹ in long term memory consolidation.

Two independent studies^{8,9} have shown that the locus coeruleus will support intracranial self-stimulation (ICSS), though with different experimental conditions no ICSS is obtained^{10,11}. There seems to be no doubt that the locus coeruleus is closely involved with the positive reinforcement system of the brain, although I agree that the details of this involvement have not been determined. My theory does not require that the locus coeruleus cells directly contracting Purkyne cells support ICSS, but only that the locus coeruleus cells receive an input from the positive reinforcement system.

My suggestions about learning in the cerebellum were made on the basis of the large amount known about cerebellar anatomy and physiology. I would expect a similar memory mechanism to operate in other parts of the brain, however, especially in view of the widespread distribution of noradrenaline nerve terminals¹². It is therefore very encouraging that a change in membrane phosphorylation as a consequence of training has been demonstrated¹³ for certain regions of the brain. The possibility that this mechanism operates in the cerebellum has certainly not been excluded by preliminary experiments on a small number of protein components of membranes from a restricted region of the cerebellum.

In conclusion I am pleased that experiments are being carried out which test my proposed memory mechanism. I would add that there is increasing support^{14,15} for the idea that the cerebellum is involved in motor learning.

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reviews

Renaissance and revolution

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The Nature of Scientific Discovery. (A Symposium Commemorating the 500th Anniversary of the Birth of Nicolaus Copernicus.) Edited by Owen Gingerich. Pp. 616. (Smithsonian Institution: Washington, DC, 1975.) \$15.

THIS misleadingly titled book is a record of a symposium held in 1973 by the Smithsonian Institute of the United States to commemorate the 500th anniversary of the birth of Copernicus. Besides the papers read at the symposium, the book includes a description of the proceedings, a large number of introductory speeches, photographs from a commemorative film made by Charles Eames, a black and white reproduction of a painting of Copernicus by Leonard Baskin and a verbatim report of the discussions which followed some of the papers. The proceedings included a performance of a musical work entitled "Copernicus—Narrative and Credo" by Leo Smit to a text written, and, in that performance, spoken, by Fred Hoyle. Neither text nor score are provided, but the text seems to have been an account of Copernicus's life and times. One of the speeches relates that Edward Rosen, who has translated some of Copernicus's major works, summed up the curiosity of Copernicus in the question "What is night, and how is it produced?". Such absurd exaggerations of the ignorance of Copernicus's contemporaries can only hinder an appreciation of his achievement. Aristotle, the prevailing authority of the time, argues for the spherical shape of the Earth from the curved edge of its shadow on the Moon during a lunar eclipse (*De Caelo*, Book II, Chapter 14).

The declared object of the symposium was to investigate the nature of scientific discovery, taking Copernicus's work as an outstanding example, rather than to clarify and evaluate that work. In fact, the papers divide into three groups, those on aspects of the history of Copernicus's times, those on the nature of scientific discovery and those providing brief expositions of questions in present-day cosmology. The first group forms the bulk of the book and contains the only papers of serious interest. The four papers on scientific discovery throw little light on



Painting of Copernicus by Jean-Leon Huens

it. Toulmin expresses his opinion that science is losing its vigour in fragmentation and technology; it should turn back to its traditional allies, cosmology and theology. Gerald Holton expounds Einstein's thoughts on scientific discovery without contributing much to the wide development and criticism which such views have received, for example from Karl Popper. Heisenberg finds the influence of tradition in research in the fact that, once one man has discovered a problem, others are attracted to the task of solving it.

Surely studies of Copernicus's own work would have provided a worthier commemoration? There is no lack of problems concerning it. Copernicus demonstrated the possibility of a radical change of viewpoint, a change which brought into prominence exactly those relationships (the relationships to the sun) which, according to Newton's gravitational theory, were of the greatest importance in explaining planetary movements. Why did Copernicus propose a change whose advantages, it seems, he could not guess at? A number of points, however, are made which illuminate Copernicus's work and its importance. Bronowski finds the inspiration for the change of viewpoint in Renaissance studies of perspective, for those studies seek rules for determining how objects in space

appear to observers situated at different vantage points. Gingerich points out the importance of Copernicus's change of viewpoint for Newton's theory. Rupert Hall stresses the mechanical problems posed by the need to reconcile the behaviour of bodies at the surface of the Earth with the Earth's motion: "If Galileo converted mechanics into a branch of natural philosophy, it was Copernicus who demanded that this be done."

The contributors are scarcely in accord over the significance of Copernicus's thought. Hall, as we have just seen, finds its value in the stimulus it gave to the investigation of real problems in dynamics. Toulmin, on the other hand, finds it in "the claim that the human mind does have the capacity to develop consistent and comprehensive theories about the world in which we live, and can do so on a scale that goes far beyond the reach of our experience." Again, whereas Bronowski finds Copernicus to have been "a humanist gentleman", inspired by the Renaissance enthusiasm for classical philosophy and literature, Toulmin finds him to be the re-incarnation of the Greek scientific spirit, that of accosting the most profound questions about the natural world. Bronowski and Toulmin differ, too, about the scientific value of humanism.

Perceptive handbook

Handbook of Perception. Volume 5: Seeing. Edited by Edward C. Carterette and Morton P. Friedman. Pp. xxii+527. (Academic: New York and London, September 1975.) \$28.50; £13.70.

THIS volume, the fourth to be published in a set of ten, could readily stand alone as an advanced textbook of visual perception. The contributors are well known, the text is uniformly clear and intelligent, and the level of presentation is more even than in earlier volumes. The organisation is conventional. Le Grand opens, with an informed history of visual science and an introduction to radiometry and photometry. In a distinguished chapter on receptive fields, J. Robson surveys the neurophysiology of the visual system and offers a shrewd discussion of the widely-canvassed idea that the visual cortex acts as an analyser of spatial frequencies. Colour vision is covered by R. and K. De Valois and by R. Boynton; temporal factors by L.

(continued from previous page)

Bronowski claims that the Renaissance study of ancient texts established standards of accuracy and exactness of inference which revealed new possibilities to science. Toulmin holds that humanism favoured a scepticism concerning human knowledge inimical to confident theorising about nature. The most fundamental disagreement, however, is that between Bronowski and Heiko Obermann. Bronowski writes, "Copernicus's attitude implies that the single truth exists in nature and as nature, and cannot be established or overturned by any authority other than the study of nature herself." Obermann does not deny that that was Copernicus's attitude; what he does deny is that it is right. "Astronomy," he asserts, "cannot reveal the 'true causes' of astral phenomena insofar as final causality lies beyond its purview." Again, "the goal of the natural sciences is validity in the sense of accuracy, whereas that of the humanities, particularly of philosophy and theology, is validity in the sense of truth." How philosophy and theology are to reveal the true causes of astral phenomena, Obermann does not proceed to explain.

The historical papers I have mentioned are interesting, as are some that I have not found room for; unfortunately they are outweighed by a mass of material which is of very limited interest. □

Ganz; spatial resolution and interaction by J. Thomas; the perception of patterns and objects by Dodwell; depth perception by W. Richards; and the perception of movement by R. Sekuler. In a final chapter on Vision and Art, M. Pirenne discusses the difference between viewing a painting and viewing a *trompe l'oeil*. Although the chapter headings are classical the fresh concepts of the seventies have a firm place in the text. If there are no traces of the grand schools that once dominated textbooks of perception and if most of the debates are now at a technical level, then that may be wholesome enough. Dodwell indeed argues here that the need for all-embracing theory of visual perception has passed and that the many different processes necessary for perception must be considered individually and in detail.

What the book lacks is an Editor and its weakness lies in the same absence of coordination that marked earlier volumes: there is clear internal evidence that the contributors have not read each other's chapters and indeed there is grave doubt as to whether anyone with editorial authority has read the entire text. Thus the concepts of Fourier Analysis are independently introduced by Robson, by Ganz and by Thomas, when what was needed was one separate account written by a single hand. It is extraordinary, even outrageous, that Boynton and De Valois have not been invited, or have not been pressed, to read each other's excellent chapters on colour vision. Both chapters, for example, discuss the colour-naming work of Boynton and the attempt by De Valois to relate Boynton's results to the electrophysiology of the primate; each refers to earlier papers by the other but there are no cross-references between the two chapters. When cross-references do occur they are often blind: thus Le Grand writes "Chemical structure of rhodopsin and of other visual pigments will be discussed by Robson, in Chapter 4 of this volume", but the reader will turn to Chapter 4 in vain. **J. D. Mollon**

Subunit interactions in enzymes

Subunit Enzymes: Biochemistry and Functions. (Enzymology: A Series of Textbooks and Monographs.) Edited by Kurt E. Ebner. Pp. x+332. (Dekker: New York, August 1975.) \$24.50.

THE aim of this book is to examine by means of representative examples the different types of subunit interactions that may exist in proteins. The proteins chosen for detailed consideration are

glutamine synthetase of *Escherichia coli*, UDP-galactose-4-epimerase, lactose synthetase, acetyl coenzyme A carboxylases, tryptophan synthetase, and the steroid carrier protein. Each is the subject of a chapter written by research workers who have made major contributions to the field. An introductory chapter, on the general structure and thermodynamics of multisubunit proteins and molecular models for cooperativity, also contains sections on haemoglobin, CTP synthetase and glyceraldehyde-3-phosphate dehydrogenases, but these are not sufficiently detailed nor up-to-date to be of value. Most of the other chapters are clear and comprehensive accounts of the relationship between structure and function, with emphasis on the significance of the multisubunit structures; some include unpublished work and addenda containing recently published findings.

The acetyl coenzyme A carboxylases of *E. coli* and avian liver are described in an excellent article by Lane, Polakis and Moss. These are examples of protomers composed of several different polypeptide chains which catalyse distinct partial reactions or, in the case of the animal enzyme, combine with the allosteric activator citrate and induce association of the protomer to form the active linear polymer. Tryptophan synthetase (Crawford) has an $\alpha_2\beta_2$ structure, and the two types of polypeptide chain again catalyse separate partial reactions; in the protomer their catalytic efficiency is increased and dissociation of the indole intermediate is prevented. Glutamine synthetase is subject to covalent modification by an elaborate enzyme cascade system, regulated by metabolites, and in the article by Ginsberg and Stadtman the interactions between adenylation, essential divalent cations and allosteric effectors are discussed in detail. Less is known about interactions between the two subunits in the UDP galactose epimerase molecule, which contains a single NAD⁺ molecule acting unusually as a prosthetic group rather than a coenzyme (Gabriel, Kalckar and Darrow). The function of α lactalbumin as a modifier protein that decreases the K_m of galactosyl transferase for glucose as acceptor, and its possible role in the control of lactose synthesis in the mammary gland, is discussed by Ebner and Magee. The squalene and sterol carrier protein is needed for maximal activity of several enzymes involved in cholesterol and bile acid synthesis, and its probable function as a lipid-carrier in these pathways is described by Dempsey.

This book serves a useful purpose in bringing together diverse examples of subunit interactions in enzymes. The omission of aspartate transcarbamylase is surprising. **K. Dalziel**

Cell membranes

Pathobiology of Cell Membranes. Vol. 1. Edited by Benjamin F. Trump and A. U. Arstila. Pp. xv+497. (Academic: New York and London, August 1975.) \$36.50; £17.50.

THIS volume is the first of a projected "multivolume treatise designed for pathologists and cell biologists interested in the role of membrane alterations in disease processes". Each of eleven chapters was written by one or more experts, and each is followed by an editors' summary. The subject matter ranges from broad topics such as "Cell Membranes and Disease Processes" (B. F. Trump and A. U. Arstila) and "Alterations in Lysosomal Membranes as Related to Disease Processes" to more technical ones, such as "The Use of Circular Dichroism in the Study of the Structure of Cell Membranes" (R. W. Henkens). In large measure, the book deals with the pathogenesis of subcellular lesions with special emphasis on the possible or probable roles of various cell membranes. Consideration is thus given to "The Cellular Mechanisms of Hormonally Induced Tissue Atrophy" (H. J. Helminen), "Thyroid Lysosomes in Health and Disease" (R. Seljelid), "Toxic Changes in Mitochondrial Membranes and Mitochondrial Function" (R. A. Goyer and B. C. Rhyne) and "Human Intestinal Epithelium as a Biological Membrane" (W. O. Dobbins III). These chapters contain much information of a correlational nature, particularly on the ultrastructural level. Other chapters give overviews of some key properties of cell membranes from physicochemical, biochemical, physiological and pathophysiological perspectives and include, in addition to the chapter on circular dichroism, the following topics: "Lipid Peroxidation and Fluorescent Molecular Damage to Membranes" (A. L. Tappel), "Colloid Osmotic Pressure as a Cause of Pathological Swelling of Cells" (J. R. Robinson), "Photopathology of the Erythrocyte Membrane" (J. S. Cook), "The Endocytic Uptake of Macromolecules" (P. J. Jacques).

All of the authors have made it clear that there are gaps between more or less defined phenomena in model membrane systems and the pathophysiological behaviour of cell membranes *in vivo*. Nowhere in biology have models been more popular than in the study of cell membranes. The search for pathogenetic mechanisms has of necessity been either empirical or reduced to narrowly defined, idealised situations. Aware of this, the editors have taken a comprehensive stance in their preface, choice of topics and comments, thus reflecting the Hegelian dictum "*das Wahre ist das Ganze.*"

The book has been written and edited with care, is beautifully illustrated and well printed. It represents a major effort to relate data and ideas about cellular membranes derived from model systems to pathobiological phenomena. It should be read by students of disease, not only to obtain information or as a guide to primary sources, but as an aid to planning experiments. I look forward to the publication of the subsequent volumes.

G. W. Richter

Microprobe analysis

Electron Microprobe Analysis. (Cambridge Monograph on Physics.) By S. J. B. Reed. Pp. xvi+400. (Cambridge University: London and New York, 1975.) £12; \$34.50.

THIS is a very useful monograph that fills a timely need for anyone who is considering the possibility of entering the field of microprobe analysis. It is but a short mental step from knowing that high energy electrons produce X rays when they strike solid objects to deciding that this would be a good method of analysing the composition of the object. The pitfalls, however, are numerous. Since the germinal work of Castaing in 1951 the field has developed at an astounding rate, and it is now known how to design suitable instruments and how to convert raw data into compositional details. Instruments are complicated and very expensive, and the investigator must often make the kinds of decisions as when he buys an automobile—which of the optional 'extras' do I really need? The analysis of raw data is equally, if not more, complex as numerous (and not small) corrections must be applied.

Clearly a readable text was badly needed to guide both neophyte and expert through this mass of detail, and this book serves that purpose well. An expert would surely conclude that each individual chapter is too short and glosses over the interesting details, but the essential facts are presented in each case even though some chapters are condensed almost to the point of reading like a telegram. To cover the fields of instrument design and the meaning of the data, the author was forced to summarise a vast literature, and he is to be commended for his efforts.

It would, however, have been valuable to provide the reader with a list of applicable texts and monographs at the end of each chapter for further reading and more detailed study. A carefully selected list would have been more valuable than chasing down the many references scattered throughout the text.

A. V. Crewe

NEW

The Nature of Seawater

Edward D. Goldberg, Editor
Scripps Institution of Oceanography

Report of the Dahlem Workshop on
The Nature of Seawater
Berlin 1975, March 10 to 15

From the Introduction by E. D. Goldberg: Herein are an unusual set of presentations. In the main they were written by some of the world's most distinguished chemists, who describe the areas of research that they are pursuing. These articles in pure chemistry were prepared for a group of marine chemists who assembled in March 1975 in Berlin, Germany to consider new approaches which might yield a better understanding of the compositions and reactions of oceanic systems. Now they are being presented to a wider audience of marine chemists with the same aim in mind.

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obituary

Robert Stoneley sometimes remarked how much he regretted never having been a research student. The First World War took him away from University, and he returned to begin teaching straight away. Yet he made valuable contributions to theoretical geophysics during the years when he was heavily engaged in lecturing, first at Sheffield then at Leeds. His published papers were short and not frequent, averaging one a year, yet he made key contributions. His first paper, in 1924, investigated the possibility of waves channelled along the welded interface of two elastic media. These came to be called Stoneley waves, though he could never bring himself to use the name and spoke of 'interface waves of Rayleigh type'.

In 1925 another important paper showed that the observations of surface waves which had been used by seismologists to calculate phase velocities in fact gave group velocities. In 1926 he made a numerical study of the Earth's elastic yielding, using C. G. Knott's velocities of sound waves and shear waves throughout the Earth. This was one of the pioneer papers that led to the conclusion that the core of the Earth is liquid.

In 1931 Stoneley explored the significance of Jeffrey's remark that a deep earthquake should excite relatively small surface waves. This consequence of dynamical reciprocity was skilfully used by Stoneley to confirm that some earthquakes, suspect on account of anomalous travel times, had indeed originated at depths of several hundred kilometres.

During the 1920s and 1930s Stoneley worked both on theoretical problems of surface wave propagation in layered

media, and on data analysis from seismograms or from tables in the International Seismological Summary. He showed how to separate double earthquakes, and how to distinguish in seismograms between Rayleigh and Love waves. Later he suggested to Michael Longuet-Higgins the work which led to an explanation of the origin of microseisms.

The Second World War, during which he carried very heavy teaching loads in Cambridge, interrupted his research for ten years. When he began to publish again it was in a valuable series of summaries or review articles on microseisms, waves in anisotropic media, surface waves, tsunami, oscillations of the Earth, deep structure of the Earth, and the history of modern seismology. He retained robust health and remarkable vigour for many years beyond retirement. During the period 1961–68 he served in Washington as consultant in the US Coast and Geodetic Survey and as Professor at the University of Pittsburgh.

A great formative influence in Robert Stoneley's career was his close friendship with Harold Jeffreys. Again and again in his papers there is reference to help or criticism from Jeffreys. Men of very different characters but similar in scientific interests—extending from differential equations to botany—their association was highly creative.

Robert Stoneley was a man of great personal kindness, responding eagerly to any request for help and going to any amount of trouble to provide it. He was patient and equitable, yet at the same time efficient, and the long list of organisations in which he served as secretary or chairman testifies to the

confidence people placed in him. His advice and warm humanity were deeply appreciated everywhere.

E. R. Lapwood

George Hoyt Whipple, whose work in the 1920s led to the control of pernicious anaemia, died on February 1 at the age of 97.

After gaining his medical degree, at Johns Hopkins, Dr Whipple remained there until, at the age of 36, he became director of the Hooper foundation for medical research at the University of California. Whilst there he demonstrated that a diet containing raw liver was an effective way of treating experimental anaemia in dogs. That discovery led to clinical trials of raw liver for various anaemias culminating in 1926 when G. R. Minot and W. P. Murphy in Boston showed that pernicious anaemia, previously incurable, could be controlled by dietary liver. Dr Whipple's contribution to that success was recognised in his sharing the 1934 Nobel prize for medicine with Minot and Murphy. It was another fourteen years before teams at Merck and Glaxo simultaneously identified vitamin B₁₂, the active factor in the liver.

In 1921 Dr Whipple moved to Rochester University where he headed the newly founded medical school. During the next thirty years, first as professor of pathology and then as dean of the medical school, he continued to study the factors, particularly iron, that can reverse experimental anaemia. He also served as a trustee of the Rockefeller Foundation from 1927 to 1943 and as a scientific director from 1936 until 1953.

announcements

International meetings

April 15, Deadline for abstracts for **Nonspecific Immune Stimulation in Cancer and Autoimmune Disease** (to be held July 5–6 in Bucharest) (2nd International Symposium on Cancer Immunotherapy, Oncological Institute, P.O. Box 5916, Bucharest 12, Romania).

April 28–30, **Land as a Waste Management Alternative**, Rochester, N.Y. (Agricultural Waste Management Conference, 207 Riley-Robb Hall, Cornell University, Ithaca, New York 14853).

Person to Person

Going on sabbatical with family to King's College, London, and would like to exchange a large 4-bedroom, furnished house in desirable area of Washington, D.C. and 20 min from NIH for comparable house or flat in interesting section of downtown London, with good schools. Seven months from June, but flexible. (Dr Mark Smulson, Department of Biochemistry, Georgetown University, Schools of Medicine and Dentistry, Washington, D.C. 20007.)

May 12–15, **Dictyostelium**, a workshop at Cold Spring Harbor, New York (Dr Harvey Lodish, Department of Biology, MIT, Cambridge, Mass, or Dr William Loomis, Department of Biology, University of California at San Diego, La Jolla, CA 92093).

May 30, Deadline for abstracts for the **27th Annual Session of the American Association for Laboratory Science** (to be held on November 7–12 at Houston) (Dr Charles W. McPherson, Animal Resources Branch, DRR, NIH, Bethesda MD20014).